Evaluating Novel Muscarinic Acetylcholine Receptor Potentiators for the Treatment of Cognitive Deficits in Schizophrenia

By

Michael D. Grannan

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Approved:

Carrie K. Jones, Ph.D.

Craig W. Lindsley, Ph.D.

P. Jeffrey Conn, Ph.D.

Ariel Y. Deutch, Ph.D.

Paul A. Newhouse, M.D.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (aka serotonin)
AChE	acetylcholinesterase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BL	Baseline
BPRS	Brief Psychiatric Rating Scale
CBV	Cerebral blood volume
CF	Conditioned fear
CNS	Central nervous system
CP	Caudate putamen
CSF	Cerebrospinal fluid
DA	Dopamine
DISC1	Disrupted in Schizophrenia-1
DNA	Deoxyribonucleic acid
EC_{50}	Fifty percent excitatory concentration
EEG	Electroencephalography
EMG	Electromyography
ERK	Extracellular receptor kinase
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid

GPCR	G protein-coupled receptor
HEK	Human embryonic kidney
HPLC-ECD	High-performance liquid chromatography with electrochemical detection
HVA	Homovanillic acid
i.p.	Intraperitoneal
IC_{50}	Fifty percent inhibitory concentration
iGluR	Ionotropic glutamate receptor
IrL	Infralimbic region of the prefrontal cortex
K^+	Potassium
kg	Kilogram
LTD	Long term depression
LTP	Long term potentiation
mAChR	Muscarinic acetylcholine receptor
mg	Milligram
Mg^{2+}	Magnesium
mGluR	Metabotropic glutamate receptor
min	Minutes
mPFC	Medial prefrontal cortex
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid

Na^{2+}	Sodium
NAS	Nucleus accumbens
NAM	Negative allosteric modulator
neo	neomycin selectable marker
ng	Nanogram
NMDA	*N*-Methyl-*D*-aspartate
NMDAR	*N*-Methyl-*D*-aspartate Receptor
No.	Number
NREM	Non-Rapid Eye Movement
PAM	Positive allosteric modulator
PANSS	Positive and Negative Syndrome Scale
PO	Per os (oral)
PV	Parvalbumin
PCP	Phencyclidine
PET	Positron emission tomography
PFC	Prefrontal cortex
phMRI	Pharmacological Magnetic Resonance Imaging
PKC	Protein kinase C
PPI	Prepulse Inhibition
PrL	Prelimbic region of the prefrontal cortex
REM	Rapid Eye Movement
sec	Seconds

SPECT	Single Photon Emission Computed Tomography
SSRI	Selective serotonin reuptake inhibitor
Temp	Temperature
Veh	Vehicle
VTA	Ventral tegmental area

CHAPTER I

INTRODUCTION

Schizophrenia

Clinical features and symptom domains

Schizophrenia is a debilitating neuropsychiatric disease affecting approximately one percent of the worldwide population irrespective of gender or race; and currently ranks as the seventh most costly medical illness to our society, making it one of the most important public health concerns in modern medicine (Freedman, 2003). While current treatments considerably reduce the suffering of the patient, approximately two-thirds of those who are diagnosed with schizophrenia require public assistance from governmental social security systems within a few years of onset (Ho et al., 1997). Traditionally, schizophrenia is defined by three distinct symptom clusters, including positive symptoms, negative symptoms, and deficits in cognition (American Psychiatric Association, 2013). Positive symptoms include auditory hallucinations, delusions (often involving paranoid beliefs that external forces are conspiring against the patient), disorganized speech or abnormal language, and disorganized behavior (American Psychiatric Association, 2013).

The negative symptoms of schizophrenia are characterized as a diminution or absence of mental functions that are normally present in healthy humans. Examples include alogia, which is a decrease in the fluency of ideas and language; affective blunting or a reduction in the intensity of an individual's emotional responses; avolition that is a decrease in the ability to initiate and pursue goal-directed activity; and

anhedonia, which involves a reduced ability to seek out and experience pleasurable activities (Andreasen, 2000; Sarkar et al., 2015). Recent epidemiological studies in schizophrenia patient populations have reported that the negative symptoms persist longer than positive symptoms and are more resistant to treatment (Boonstra et al., 2012; Chang et al., 2011). Moreover, the severity of negative symptoms in individuals with schizophrenia has been shown to be a better predictor of concurrent and future socio-occupational functioning and potential conversion to psychosis in patients with at risk mental states (Rabinowitz et al., 2012; Kurtz et al., 2005; Milev et al., 2005; Lencz et al., 2004; Valmaggia et al., 2013).

Cognitive dysfunction in patients with schizophrenia may present as attentional impairment, deficits in working and episodic memory functions, and disruptions in executive function, and have been extensively described in the CATIE trials (Clinical Antipsychotic Trials of Intervention Effectiveness) (Keefe et al., 2006) and through the MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) initiative (Buchanan et al., 2007). Many of these cognitive domains are impaired prior to the emergence of the positive symptoms and detectable at the time of the first psychotic episode (Bilder et al., 2000; Davidson et al., 1999). In recent years, interest in the cognitive deficits has dramatically increased with reports that they are consistently more reliable predictors of functional outcomes (Green et al., 2000). With this in mind, there has been a push towards developing novel antipsychotic agents that exhibit a broader range of efficacy across the three major symptom clusters, including the cognitive deficits as well as the negative symptoms as mentioned earlier.

As shown in Figure 1, the time course for the disease progression of schizophrenia is often associated with various prodromal symptoms and behaviors, including cognitive deficits (McGlashan, 1996; Gottesman & Erlenmeyer-Kimling, 2001), followed by the onset of the positive and negative symptoms in late adolescence or early twenties (Hafner et al., 1994; Heilbronner et al., 2016; Lewis & Lieberman, 2000; Talonen et al., 2016). Since the initial characterization and description of schizophrenia, patients with the disease have, in large part, been treated as a homogenous population, with little work done to differentiate stages of progression. There is emerging evidence, however, to support a phenotypic progression throughout the life of a patient with schizophrenia thought to be due in part to changes in neural circuitry and/or responses to lifelong antipsychotic therapies (see Figure 1). For example, meta-analyses report decreases in the integrity of grey and white matter (Chan et al., 2011; Mathalon et al., 2001) and faster rates of glutamate signaling decline in patients with schizophrenia, compared to healthy aging individuals (Marsman et al., 2013). Additionally, structural magnetic resonance imaging (MRI) studies report a significant reduction in brain tissue volume in patients with schizophrenia that has subsequently been shown to be progressive in several longitudinal studies (Borgwardt et al., 2008; Cahn et al., 2002; DeLisi, 2008; Heilbronner et al., 2016; Hulshoff Pol & Kahn, 2008; Pantelis et al., 2003; van Haren et al., 2008). This may be due to reduced neuropil, suggesting changes in synaptic, dendritic, and axonal organization, and is observed most prominently in the prefrontal cortex (PFC) (Harrison, 1999). For these reasons, one of the prevailing hypotheses regarding the underlying pathophysiology of schizophrenia suggests that these structural changes result in subsequent progression of abnormalities in the normal

functional connections between different brain regions, particularly the PFC (Cho et al., 2006; Cole et al., 2011; Van Snellenberg et al., 2006). For example, functional MRI studies examining patients in the early stages of the illness have shown patterns of hyperconnectivity between the PFC and hippocampal with other brain regions, which appear to diminish with the progression of the illness (Marsman et al., 2013; Schobel et al., 2013; Sun et al., 2013). Therefore, it is reasonable to suggest that early stages of the disease are associated with a shift in the excitation:inhibition balance in critical PFC circuits, consistent with models of disinhibition (Uhlhaas, 2013). Thus, stratifying patients based on their stage of illness will be of utmost importance when designing animal models of the disease, assessing novel therapeutics, and carrying out future clinical trials.

Clinically available typical (eg, haloperidol) and atypical (eg, clozapine, risperidone) antipsychotic medications reliably treat the positive symptoms, but have little or no effect on the negative symptoms or cognitive impairments (Keefe et al., 2007; Swartz et al., 2008), and will be discussed at length in future sections. While the etiology of schizophrenia is unknown, there is mounting evidence that the pathophysiology of the illness is linked to disruptions in normal signaling of several different neurotransmitter systems, including dopaminergic, glutamatergic, GABAergic, serotonergic and cholinergic systems (Carlsson, 1977; Guan et al., 1999; Krystal et al., 2002; Lewis & Moghaddam, 2006; Scarr et al., 2007). As background for my thesis work, I will focus on the delicate interplay between the dopaminergic and glutamatergic systems, and how selective modulators of muscarinic acetylcholine receptors affect each at length in this chapter.

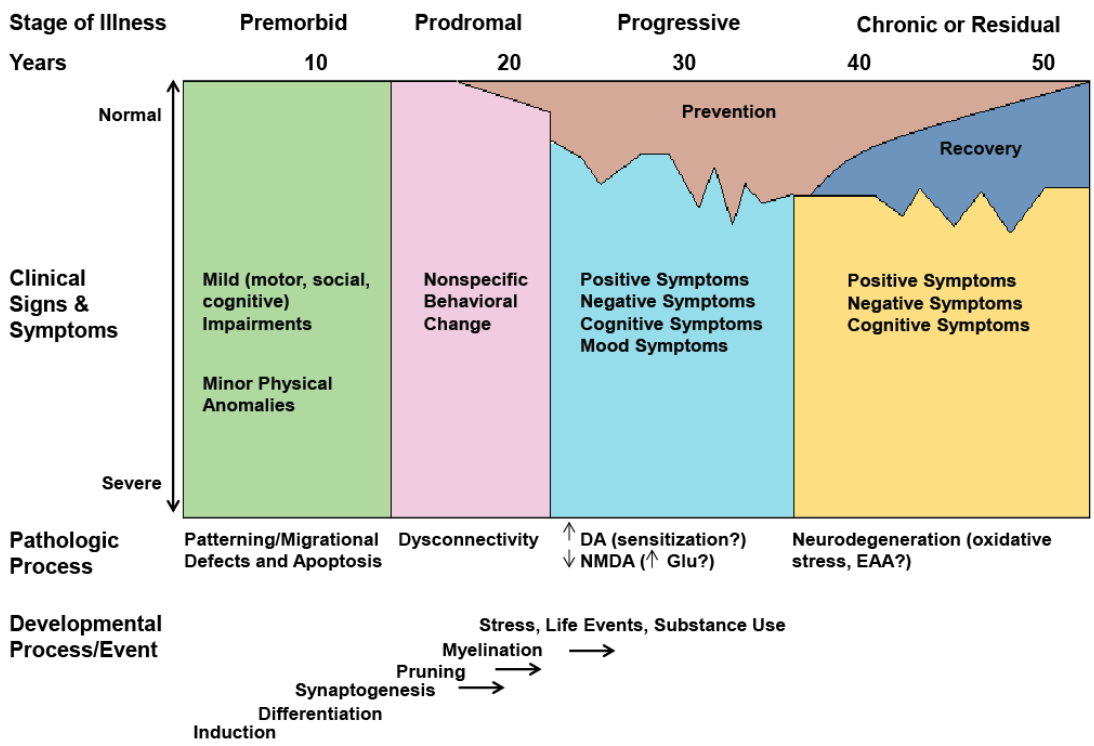


Figure 1. Time course of schizophrenia disease progression. The diagram above depicts the clinical signs and symptoms observed in patients from the premorbid stage of the illness through the chronic stages. Accompanying these symptoms are proposed pathological processes that coincide with developmental events throughout the life of a patient with schizophrenia. Adapted from (Lewis & Lieberman, 2000).

Dopamine hypothesis

Aberrant dopamine (DA) signaling has been implicated in schizophrenia for over 50 years and is the principal explanatory model of antipsychotic drug action. This hypothesis theorizes there is an excessive amount of dopaminergic signaling in subcortical structures of the brain which leads to over activation of DA D2 receptors. The advent of antipsychotic agents that increased the metabolism of DA when administered to animals suggested a vital role for dopamine signaling in psychosis (Carlsson & Lindqvist, 1963). Furthermore, another antipsychotic agent, reserpine, was found to prevent the reuptake of DA leading to decreased dopaminergic signaling (Carlsson et al., 1957). Conversely, drugs that act to increase synaptic DA levels in the brain can recapitulate psychotic symptoms in animals and humans (Lieberman et al., 1987). The role of DA in relation to antipsychotic agents became clear with the finding that effectiveness in the clinic was directly related to the drug's ability to inhibit DA receptors (Creese et al., 1996; Seeman & Lee, 1975).

It was not until the 1980s that investigation began into regional specificity of dysfunctional DA signaling and the distinct subtypes of dopamine receptors involved. These studies originated as a result of clinical findings from post-mortem and cerebrospinal fluid (CSF) experiments that revealed conflicting data for a homogenous DA excess theory in schizophrenia (Bird et al., 1977; Crow et al., 1979; Widerlov, 1988). Specifically, these early post-mortem studies indicated patients with schizophrenia had increases in striatal DA levels that were accompanied by increases in DA D2 receptor density (Mackay et al., 1982; Owen et al., 1978). More recent studies have suggested an increase in the capacity to produce DA in the terminals of substantia nigra neurons in

patients with schizophrenia, as measured by an increase in the rate-limiting enzyme tyrosine hydroxylase (Howes et al., 2013).

The development of *in vivo* quantification techniques, such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT), allows for measurement of DA synthesis, DA release, and the availability of postsynaptic DA receptors (Kim et al., 2013). Taking advantage of these techniques, studies have shown that patients diagnosed with schizophrenia show an increase in the uptake of [18F] DOPA, an L-DOPA PET tracer, in the striatum, which indirectly indicates an increase in striatal dopamine signaling (Egerton et al., 2010). Additionally, studies examining cortical blood flow in drug-naïve patients with schizophrenia reveal a significant reduction in perfusion in lateral, orbital and medial prefrontal cortices (Andreasen et al., 1997). Taken together, these data point towards regional brain dysfunction in schizophrenia that constitutes hyperdopaminergic signaling in subcortical regions and hypodopaminergic signaling in cortical regions.

Two important components of the dopaminergic system are the mesolimbic and mesocortical pathways. Two key parts of the these two DA pathways have been implicated in the pathophysiology of schizophrenia, including the dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAS), and the dopaminergic neurons of VTA neurons projecting to the PFC (Figure 2). Preclinical animal studies that lesioned prefrontal cortical DA neurons resulted in increased release of DA and increased expression of DA D2 receptor in the nucleus accumbens (Pycock et al., 1980). Conversely, local infusion of DA agonists directly to the PFC results in decreased DA metabolite levels in the striatum (Scatton et al., 1982). These data provide

direct evidence for cortical dopaminergic control of subcortical DA signaling. Furthermore, examples of human and animal frontal lobe lesions result in strikingly similar phenotypes to those characterized as negative symptoms in schizophrenia (Mesulam, 1986; O'Driscoll & Leach, 1998), and longitudinal studies of plasma homovanillic acid (HVA) levels in patients receiving antipsychotic treatment relate directly to efficacy on the positive symptoms (Pickar et al., 1986).

The DA hypothesis of schizophrenia has evolved since its inception nearly 60 years ago from a theory of global dopaminergic dysregulation to one of regional specific dysfunction. It is now clear that subcortical hyperdopaminergic signaling in mesolimbic projections leads to overstimulation of DA D2 receptors and is responsible, in large part, for the positive symptoms associated with the disease. In contrast, hypoactive mesocortical dopaminergic projections lead to hypostimulation of cortical DA dopamine D_1 receptors (D_1) receptors and is heavily implicated in both the negative symptoms and cognitive deficits observed in schizophrenia. Moreover, postmortem studies of patients with schizophrenia have revealed regions of PFC pyramidal cell dendritic atrophy thought to result in decreased cortical DA levels and possible changes in DA D_1 receptors (Arnsten et al., 2016). It is also clear that these two pathways are strongly linked, as a deficiency in mesocortical DA signaling results in disinhibition of mesolimbic dopaminergic activity, which has led to a revised DA hypothesis for the pathophysiology of schizophrenia (Davis et al., 1991; Deutch, 1992). To date, alterations in DA function are the most understood and directly linked to manifestations of the symptoms of schizophrenia, but much work needs to be done to understand the interplay between the DA system and glutamatergic and GABAergic systems. It is likely that disruptions in

normal signaling in these systems could lead to or be associated with aberrant cortical and subcortical DA control.

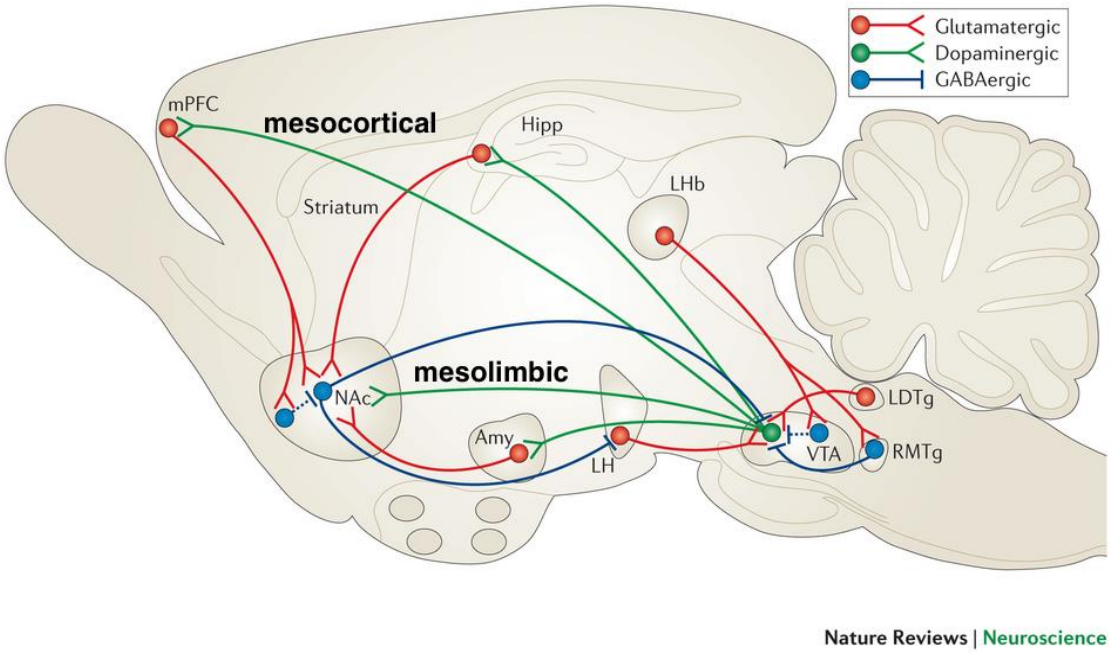


Figure 2. Mesocortical and mesolimbic circuitry in the rat brain. It is hypothesized that hyperdopaminergic signaling in the mesolimbic circuit accounts for the positive symptoms of schizophrenia, while a hypodopaminergic state in the mesocortical circuit leads to negative symptoms and cognitive deficits. This may also be referred to as the hypofrontality model of schizophrenia. Figure adapted from (Russo & Nestler, 2013).

NMDA receptor hypofunction hypothesis

Within the mammalian central nervous system, glutamate is the primary excitatory neurotransmitter and is responsible for the generation of fast excitatory synaptic responses at most central nervous system (CNS) synapses (Dingledine et al., 1999). Ionotropic glutamate receptors, including the N-methyl-*D*-aspartate (NMDA) receptor subtype, are a well characterized family of glutamate receptor cation channels that are responsible for mediating fast synaptic responses at glutamatergic synapses (Javitt, 2010). The NMDA receptor is normally composed of two obligatory NR1 subunits and two NR2A-D subunits, though a related gene family, termed NR3A or NR3B, may also be present and exert an inhibitory effect on receptor activity. Functional heterotetramers maintain a voltage-sensitive block through extracellular Mg^{2+}, which prevent cation flow at resting membrane potentials. NMDA receptors also require one of two co-agonists to allow for channel opening, either glycine or D-serine (Kleckner & Dingledine, 1988). Upon depolarization, and in the presence of co-agonist, the Mg^{2+} block is dislodged and a voltage-dependent flow of Na^{+} and Ca^{2+} enter the cell (Cull-Candy et al., 2001; Paoletti & Neyton, 2007). NMDA receptor activation, in particular through induction of synaptic plasticity, is known to be a requisite component of long and short-term memory, memory consolidation, spatial memory, episodic memory, and contextual fear memory (Tsien et al., 1996; Zhao et al., 2005). It is clear that with such vital roles in normal brain function, perturbation of this system may have strong implications for many neuropsychiatric diseases.

There is growing evidence that modulation of NMDA receptors is involved in the pathophysiology of the psychotic symptoms and cognitive impairments of many

neuropsychiatric disorders such as schizophrenia (Konradi & Heckers, 2003). This hypothesis stemmed from observations that non-competitive NMDA receptor antagonists, such as phencyclidine (PCP) and ketamine, produce symptoms in humans that mimic the positive symptoms (delusions, hallucinations, thought disorders), negative symptoms (alogia and anhedonia), and cognitive deficits (attention and working memory) observed in schizophrenia (Krystal et al., 1994). Moreover, in many clinical cases, there are not sufficient assessments available to distinguish patients on PCP from those with acute functional mental illness (Yesavage & Freman, 1978). Additionally, administration of either PCP or ketamine results in the precipitation of symptoms in patients that were previously stabilized on antipsychotic medication (Bakker & Amini, 1961; Ban et al., 1961; Cohen et al., 1962; Davies & Beech, 1960; Lahti et al., 1995; Luby et al., 1959). However, the precise mechanism of action for PCP-induced brain disruptions was not determined until the pioneering work by David Lodge and colleagues, which linked the blockade of NMDA receptors directly with the behaviors observed following PCP administration (Lodge & Anis, 1982).

Previous reports indicate that blocking NMDA receptors *in vivo* leads to excessive release of glutamate (Adams & Moghaddam, 1998; Moghaddam et al., 1997) and acetylcholine (ACh) (Giovannini et al., 1994; Hasegawa et al., 1993; Kim et al., 1999) in the cerebral cortex. In animal studies, prolonged exposure to glutamate and ACh has been associated with irreversible morphological changes in cortical neuron populations (Olney & Farber, 1995). In rats, prolonged exposure to high doses of NMDA receptor antagonists result in irreversible damage and neuron death in many cortical and limbic regions of the brain (Corso et al., 1997). It is postulated that excessive release of

excitatory neurotransmitters could lead to overstimulation of postsynaptic neurons, which causes these morphological changes, and may account for the cognitive and behavioral disturbances observed when NMDA receptor antagonists are administered (Olney & Farber, 1995).

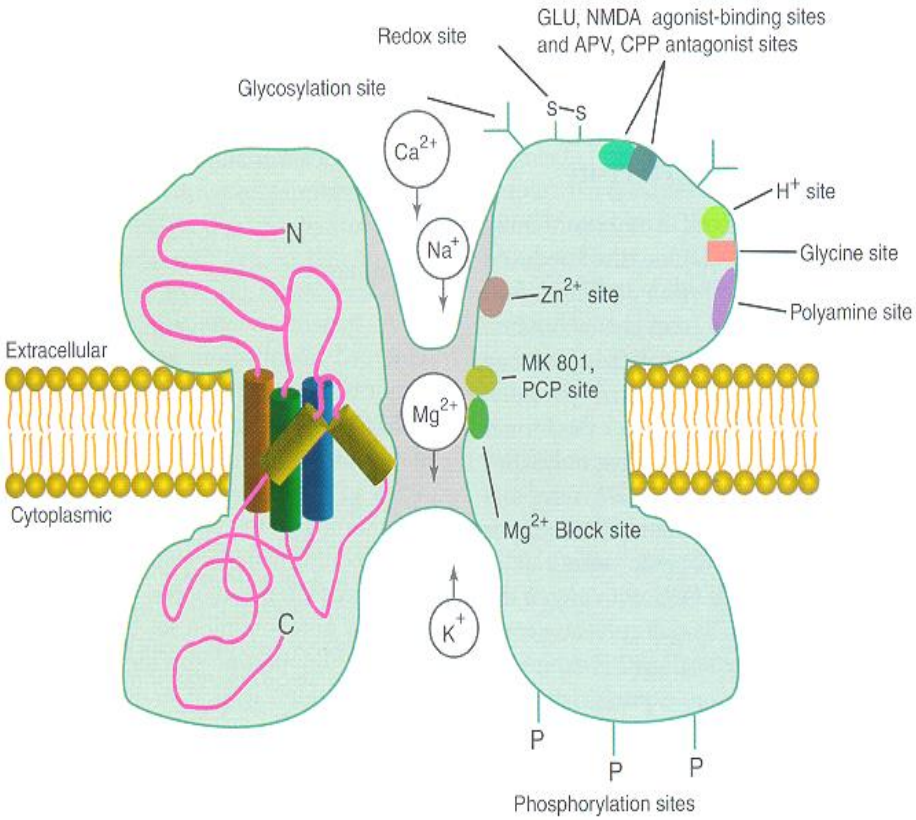


Figure 3. NMDA receptor. Adapted from (Waxham, 2004). NMDA receptors have been implicated in the etiology, or as a result, of the schizophrenia disease state. The schematic shows a functional NMDA receptor with binding sites for agonists, co-agonists, and antagonists. Also depicted are the permeable cations and possible glycosylation and phosphorylation sites.

Electron microscopy studies carried out on post-mortem tissue from patients with schizophrenia report consistent pathological abnormalities compared to healthy human controls. Frontal and limbic cortices exhibit dystrophic changes that include pronounced swelling in dendrites with reduced number or absent microtubule, dense and degenerating presynaptic endings, and pathological changes in myelinated fibers (Uranova, 1988). Other reports indicate a 30% reduction in the size of spines in the basal ganglia (Roberts et al., 1996). In cortical tissue, Golgi staining revealed decreases in neuropil, dendritic complexity of pyramidal neurons, and spine density (McGlashan & Hoffman, 2000; Selemon et al., 1995). Many, if not all, of the pathological changes observed in human tissue can be recapitulated in mouse models of NMDA receptor hypofunction. In mice lacking sufficient levels of the co-agonist D-serine, reductions in the complexity, total length, and spine density of pyramidal cells has been observed, along with decreases in neuropil and cortical volume (Balu et al., 2012). In mice expressing only 10% of normal NMDA receptor levels, dendritic spine number and expression of Disrupted in Schizophrenia-1 (DISC1), a protein involved in embryonic and adult neurogenesis, is reduced in an age-dependent manner. Interestingly, sub-chronic administration of an NMDA receptor antagonist produces similar synaptic reductions in spine density and DISC1 (Ramsey et al., 2011). These findings, among others, provide significant validity to the role of NMDA receptor hypofunction in morphological changes observed in patients with schizophrenia.

It is well established that NMDA receptor hypofunction leads to excessive neurotransmitter release at glutamatergic synapses through disinhibition of pyramidal cells. Early studies in the CA1 region of the hippocampus revealed that GABAergic

interneurons were 10-fold more sensitive to NMDA receptor antagonists than pyramidal cells in the same region (Grunze et al., 1996). Additional studies utilizing *ex vivo* electrophysiology showed that NMDA receptors provide excitatory drive on GABAergic interneurons in the olfactory bulb, posterior cingulate and retrosplenial cortex (Li et al., 2002; Zhang et al., 2008). Thus, administration of NMDA receptor antagonists, or under expression of NMDA receptors, results in disinhibition of glutamatergic neurons through loss of recurrent inhibition provided by GABAergic neurons. In accordance with these findings, deficits in GABAergic signaling have been reported in the cortex and limbic system of patients with schizophrenia (Schwarcz et al., 2001). Immunohistochemical studies of recurrent inhibitory interneurons revealed that a subset containing parvalbumin (PV) are particularly vulnerable to disruption in schizophrenia (Beasley & Reynolds, 1997). Pre-clinical studies examining the effect of NMDA receptor antagonists in mouse cortical tissue reveal similar reductions in PV expression (Behrens et al., 2007; Zhang et al., 2008). Taken together, these findings indicate a vital role for NMDA receptors on GABAergic interneurons and provide a viable mechanism for many of the neurochemical, electrophysiological, and behavioral changes observed in models of the disease, and in the disease itself.

Genetic models of NMDA receptor hypofunction have been developed to further elucidate the role of aberrant glutamatergic signaling in animal models of disease. One of the first models described was the NR1 knockdown model of NMDA receptor hypofunction (Mohn et al., 1999). Since complete deletion of *Grin1*, the gene encoding the obligatory NR1 subunit of the NMDA receptor, results in lethal respiratory failure (Forrest et al., 1994; Li et al., 1994), this model employs only a partial loss-of-function,

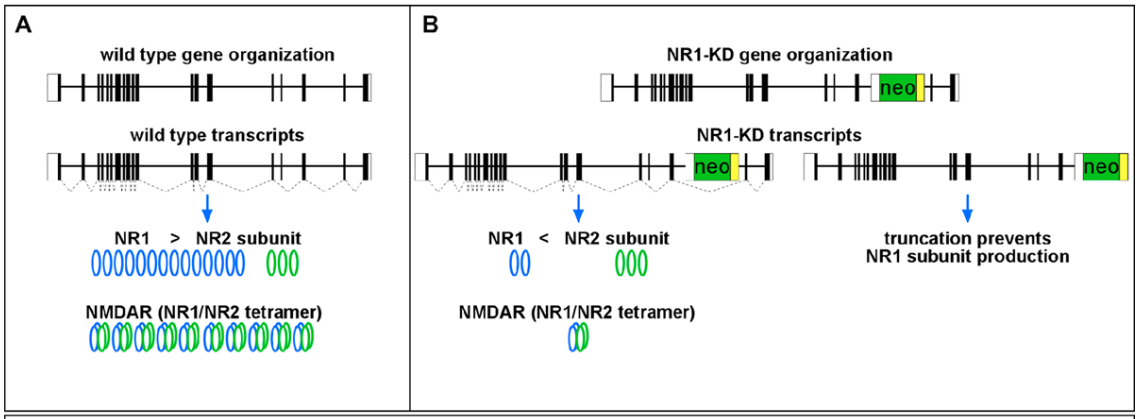
or hypomorphic, mutation. This is achieved by targeted insertion of a 2 kilobase pair segment of foreign DNA, a neomycin selectable marker (*neo*), into the *Grin1* gene near the 3' end. The presence of this foreign DNA causes premature termination in the majority of transcripts at the polyadenylation sequence of *neo*, resulting in a reduction in the amount of full-length mRNA transcript encoding the NR1 subunit. Splicing removes a portion of the *neo* sequence, resulting in sufficient quantities of functional NR1 subunits to allow for NMDA receptor formation, though at a greatly reduced level (Figure 4). As a consequence, there are sufficient functional NMDA receptors expressed to support survival of the animal, but with only 10-20% of wildtype controls in critical brain regions implicated in schizophrenia, as measured in radioligand binding studies utilizing [^{3}H] MK-801 (Duncan et al., 2002; Mohn et al., 1999) (Figure 4).

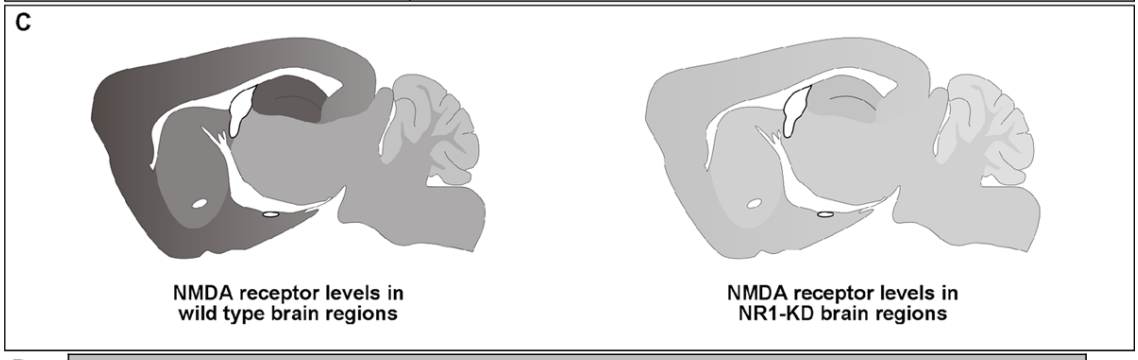
NR1 knockdown (KD) mice exhibit abnormal behaviors consistent with acute NMDA receptor antagonist administration in several assays used to evaluate new antipsychotic medications. For example, NR1 KD mice show increases in spontaneous locomotor activity and stereotypic behavior, and decreases in habituation to novel environments, sensorimotor gating, sociability, and performance in spatial and non-spatial working memory tasks (see Table 1). Many of these phenotypes are only observed in global NR1 KD animals, though there is a growing body of literature characterizing the effects of cell type-specific ablation, as well as deletion of NR2 subunits (Belforte et al., 2010; Rampon et al., 2000; Tsien et al., 1996; von Engelhardt et al., 2008; Zhao et al., 2005). Consistent with MK-801 and PCP-treated mice, currently available typical and atypical antipsychotics can attenuate some of the behavioral deficits observed in NR1 KD mice, such as increased locomotor activity (Duncan et al., 2006; Mohn et al., 1999;

Ramsey et al., 2008). While this adds to the validity of the model, these findings would suggest an interaction between antipsychotic agents and neurotransmitter systems that are known to regulate locomotor activity in rodents, namely the dopaminergic system. However, initial characterization of the dopaminergic system in NR1 KD mice would suggest a lack of excessive signaling, as observed by unchanged dopamine D1 and D2 receptor expression level, analogous behavioral responses to dopaminergic agonists or amphetamine, and similar levels of amphetamine-induced dopamine efflux compared to wildtype animals (Mohn et al., 1999; Ramsey et al., 2008). As such, there is a disconnect between the observed effects with current antipsychotics and the construct validity of the NR1 KD model when assessing changes in locomotor activity. One proposed explanation suggests that the observed locomotor abnormalities in NR1 KD mice may reflect a deficit in cognition rather than excessive dopamine signaling (Nilsson et al., 2001). With this in mind, it will be important to evaluate novel therapeutics in NR1 KD mice using assays that require intact signaling in brain regions known to be associated with the cognitive deficits and negative symptoms of schizophrenia.

Unlike acute pharmacological models of NMDA receptor hypofunction, NR1 KD mice demonstrate deficits in several models predictive of antipsychotic-like activity without non-competitive NMDA receptor antagonists present. This may be critical, as will be discussed in future chapters, since novel mechanisms for alleviating NMDA receptor hypofunction may rely on potentiating NMDA current. Also of importance, NR1 KD mice experience NMDA receptor hypofunction during critical developmental stages that may be relevant to schizophrenia disease populations, something that most acute or chronic pharmacological models do not account for. Taken together, the NR1 KD mouse

model of NMDA receptor hypofunction represents an innovative approach for assessing modulation of the glutamatergic system using novel therapeutic mechanisms.





D

Brain Region	WT MK-801 Bmax	NR1 KD MK-801 Bmax	% NMDA Receptor Expression
Hippocampus	1970±62 fmol/mg	207±73 fmol/mg	10% of WT
Frontal Cortex	1320±82 fmol/mg	187±54 fmol/mg	14% of WT
Striatum	780±11 fmol/mg	165±16 fmol/mg	21% of WT
Brainstem	120±15 fmol/mg	48±10 fmol/mg	40% of WT

Figure 4. Creation of NR1 KD mouse model of global NMDAR hypofunction. (A) *Grin1* is transcribed and alternately spliced, resulting in NR1 subunits that are produced in excess of NR2 subunits. In this case, expression of NR2 subunit is the limiting factor for production of functional NMDA receptors. (B) In NR1 KD mice, the presence of *neo* results in a high occurrence of truncated transcripts, though proper splicing does allow for the production of functional NR1 transcripts, though at a much lower level than in wildtypes. NR1 subunit expression is no longer in excess of NR2 subunits, and becomes the limiting factor in functional NMDA receptor expression. (C) Hypothetical illustration depicting relative levels of NMDA receptor expression, where darker shades represent higher levels of the receptor. (D) Initial findings reported by (Mohn et al., 1999) using radioligand binding to quantify maximal binding (Bmax) of MK-801. (adapted from (Ramsey, 2009)).

Phenotype	Knockdown Location	Reference
↓ Radial arm maze performance	Global NR1 KD	(Dzirasa et al., 2009)
↓ Attentional modulation of evoked potentials	Global NR1 KD	(Bickel et al., 2007)
↓ Social interaction	Global NR1 KD	(Duncan et al., 2004; Halene et al., 2009; Mohn et al., 1999)
↓ Y-maze performance	50% in hippocampal and cortical interneurons	(Belforte et al., 2010)
↓ Social recognition	50% in hippocampal and cortical interneurons	(Belforte et al., 2010)
↓ Morris water maze performance	Hippocampal and forebrain NR2B	(Rampon et al., 2000; Tsien et al., 1996; von Engelhardt et al., 2008)
↓ Novel object recognition	Hippocampal NR1 deletion	(Rampon et al., 2000; Tsien et al., 1996; von Engelhardt et al., 2008)
↓ Radial arm maze performance	NR1 in CA1 and dentate gyrus	(Niewoehner et al., 2007)
↓ Contextual fear memory performance	Cortical NR2B deletion	(Zhao et al., 2005)
↑ Spontaneous locomotor activity	Global NR1 KD	(Mohn et al., 1999)
↓ Sensorimotor gating (PPI)	Global NR1 KD	(Duncan et al., 2006; Duncan et al., 2004; Fradley et al., 2005)
↓ Auditory ERP gating	Global NR1 KD	(Halene et al., 2009)
↓ Visual ERP gating	Global NR1 KD	(Halene et al., 2009)

Table 1. Effects of global and cell type-specific knockdown or knockout of NMDA receptor subunits in mouse models of schizophrenia.

Current antipsychotic therapies

First-generation, also referred to typical, antipsychotics, target the DA D2 receptor with high affinity where they act as antagonists to block excessive DA signaling. While these treatments are very effective at treating the psychotic symptoms of the disease, they have high rates of extrapyramidal side effects such as tardive dyskinesia due to excessive inhibition of DA receptors in the basal ganglia, and do not address disturbances observed in the negative symptoms or cognitive deficits of patients with schizophrenia (Gerlach et al., 1975; Miyamoto et al., 2005). In addition, 30% of all patients with schizophrenia remain treatment resistant to typical antipsychotics, such as haloperidol and chlorpromazine, and up to 50% only have a partial response (Fleischhacker, 1995; Kane, 1989). These medications also possess some affinity for muscarinic, histaminergic, and noradrenergic receptors, which could contribute to overlapping adverse effects, such as cognitive deficits and sedation (Hill et al., 2010).

The development of second-generation, or atypical, antipsychotics promised to improve therapeutic effects and decrease the side effects associated with typical antipsychotics. However, all atypical antipsychotics still share DA D2 receptor antagonism properties, albeit with much lower binding affinity. In addition to DA D2 receptor activity, atypical antipsychotics exhibit a diverse pharmacology with appreciable binding affinities towards dopaminergic, serotonergic, histaminergic, muscarinic, and adrenergic receptors, along with activity at many transporters found in the CNS (Kapur et al., 2002; Meltzer, 1989; Schotte et al., 1996; Schotte et al., 1993). Clozapine, marketed under the name Clozaril®, remains the gold-standard antipsychotic for treatment-resistant

patients, though the clinical superiority remains unclear for treatment of the psychotic symptoms (Leucht et al., 1999; Markowitz et al., 1999). Clozapine also carries a significant risk of adverse side effects, most notably agranulocytosis, which requires the patient to undergo frequent monitoring of leukocyte levels (Honigfeld et al., 1998). The risk for adverse side effects spurred the development of additional atypical antipsychotics, such as risperidone (Risperdal®), olanzapine (Zyprexa®), quetiapine (Seroquel®), and ziprasidone (Geodon®) (Lieberman et al., 2005). These do not carry the risk of agranulocytosis, but pose problems in that they increase the risk of causing weight gain and disturbances in glucose and lipid metabolism (Bak et al., 2014).

As stated previously, clozapine carries a significant side effect profile risk that may preclude use in some individuals. The risk for serious and sometimes life threatening side effects makes clozapine a poor candidate for a first-line drug in many patients. Treatment with clozapine often begins only after a lack of adequate response with other typical or atypical antipsychotics or in cases where severe side effects are observed with other medications. Clozapine is the only antipsychotic to consistently show efficacy in patients with treatment refractory schizophrenia, making it the only option for some patients (Chakos et al., 2001; Kane et al., 1988). That being said, patients who are stably controlled on other antipsychotics may see additional benefits when taking clozapine, such as return to full time employment, which make the risk of severe side effects justifiable. In some cases, highly dysfunctional patients (those suffering from behavioral outbursts or those with suicidal intent, etc.) may only respond to clozapine (Buchanan et al., 1998).

The effects that atypical antipsychotics have on the negative symptoms and cognitive deficits have been controversial and inconsistent (Keefe et al., 1999; Meltzer & McGurk, 1999; Worrel et al., 2000). The improvements reported in the negative symptom domain are usually moderate to small compared to typical antipsychotic or placebo control groups, and often times the impact on specific components are not stated (Goff & Evins, 1998; Leucht et al., 1999; Marder et al., 1997). One theory for the observed decrease in negative symptoms points solely to the decrease in extrapyramidal side effects or other symptoms that were typically observed in patients prescribed typical antipsychotics (Carpenter et al., 1995; Marder & Meibach, 1994; Meltzer, 1995; Remington & Kapur, 2000). Likewise, it is unclear whether efficacy of atypical antipsychotics on cognitive performance is linked to improvement of positive symptoms (Buckley, 2001). Several studies have reported modest improvements in cognition in patients taking atypical antipsychotics, when compared to typical antipsychotic controls. These improvements include efficacy on tests of verbal fluency, fine motor function, and executive function, with improvements in learning and memory (Keefe et al., 1999; Meltzer & McGurk, 1999; Worrel et al., 2000). Taken together, it is clear there is a large unmet medical need for therapies that address all three symptom domains of schizophrenia without the high risk of dose-limiting side effects.

Table 2. Summary of current antipsychotic mechanisms and their functional outcomes in patients. Adapted from (Miyamoto et al., 2012).

Mechanisms	***Potential clinical efficacy***	***Potential consequences***
D_2 receptor antagonism	↓ positive symptoms	Extrapyramidal side effects
		↑ Negative symptoms
		↑ Cognitive symptoms
D_2 receptor partial agonism	↓ positive symptoms	↓ Extrapyramidal side effects
	↓ negative symptoms	Behavioral activation
	↓ cognitive symptoms	
↑ DA and NE release in the PFC	↓ negative symptoms	Behavioral activation
	↓ cognitive symptoms	
	↓ depressive symptoms	
ACh release in the PFC	↓ cognitive symptoms	
5-HT_{2A} antagonism	↓ negative symptoms	↓ Extrapyramidal side effects
5-HT_{1A} partial agonism	↓ negative symptoms	
	↓ cognitive symptoms	
	↓ anxiety symptoms	
	↓ depressive symptoms	
Muscarinic receptor antagonism	↓ EPS	↑ Anticholinergic symptoms
Muscarinic receptor agonism	↓ positive symptoms	
	↓ cognitive symptoms	
Glutamate modulation	↓ positive symptoms	
	↓ negative symptoms	
	↓ cognitive symptoms	
	↓ illness progression	

Novel approaches to treating schizophrenia

Muscarinic Acetylcholine Receptor (mAChR) Targets

The inability of current antipsychotics to address the positive, negative, and cognitive symptoms associated with schizophrenia concurrently, and with few side effects, has led to research into novel therapeutic mechanisms. One such target that has garnered interest in the last 20 years is the muscarinic acetylcholine receptor (mAChR) system, which has several lines of evidence implicating it in the etiology of schizophrenia and other psychiatric illnesses. The mAChRs are G-protein coupled receptors (GPCRs) for the neurotransmitter acetylcholine (ACh) and consist of five different subtypes, termed M_1-M_5 (Bonner et al., 1987). Each of the five mAChR subtypes is a seven-transmembrane (TM) protein that can be further divided into two major functional classes based on G-protein coupling. The M_1, M_3, and M_5 receptor subtypes couple to $G\alpha_q$ proteins, resulting in the activation of phospholipase Cβ and the subsequent release of calcium from intracellular stores and stimulation of protein kinase C and resultant intracellular signaling pathways. The M_2 and M_4 receptor subtypes couple predominantly to $G\alpha_{i/o}$ proteins to inhibit adenylate cyclase, causing a decrease in intracellular cAMP levels and overall decrease in cellular excitability. The M_1, M_4 and M_5 receptor subtypes are predominantly expressed in the CNS, while the M_2 and M_3 receptor subtypes are widely distributed in both the CNS and peripheral tissues (Caulfield & Birdsall, 1998; Wess et al., 2007). There is significant sequence homology at the orthosteric ACh binding site across all of the subtypes of mAChR, which has made the development of subtype-selective ligands for muscarinic receptors difficult.

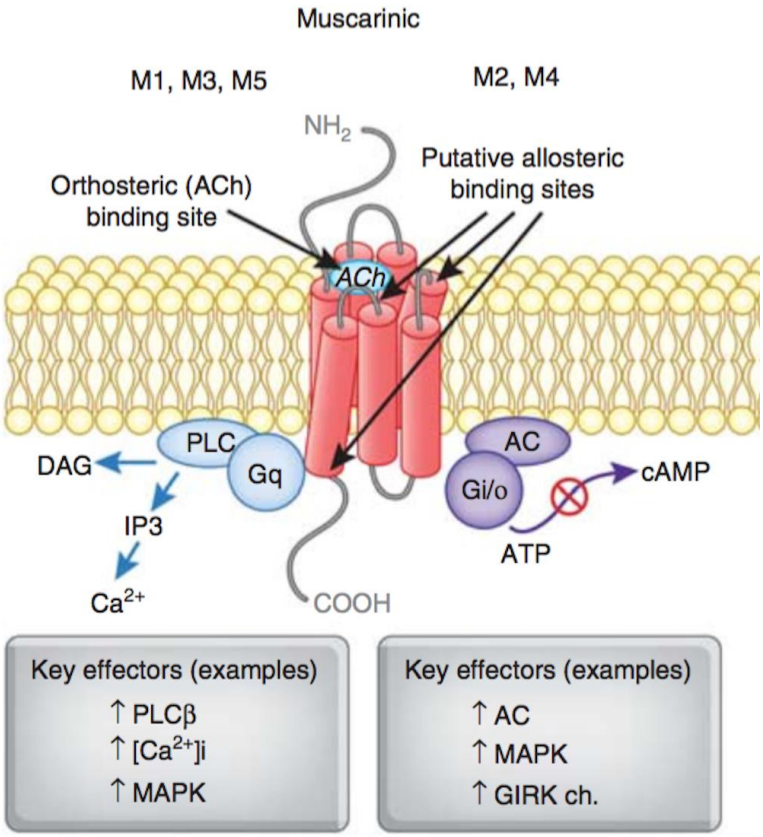


Figure 5. Muscarinic Acetylcholine Receptor Subtypes. The mAChR M_1, M_3, and M_5 subtypes are G_q coupled and activation leads to increased neuronal excitability through increases in intracellular calcium and activation of MAPK and PLC. M_2 and M_4 subtypes are G_i/G_o coupled and activation leads to inhibition of adenylyl cyclase and decreased excitation of the cell. Figure from (Jones et al., 2012).

Previous work has shown that mAChRs are vital for modulation of multiple functions in the CNS, including cognition and motor control. Clinical literature indicates that impairment of cholinergic transmission contributes to the cognitive deficits in Alzheimer's disease and other neurologic disorders (Auld et al., 2002; Winkler et al., 1998). Additionally, multiple clinical studies indicate agents that enhance cholinergic transmission, including acetylcholinesterase (AChE) inhibitors, are clinically efficacious in reducing the loss of cognitive function in patients with Alzheimer's disease and other memory disorders (Davis et al., 1992; Langmead et al., 2008; Rogers et al., 1998). However, the efficacy of these drugs in enhancing cognition is limited, in part because AChE inhibitors increase transmission at all cholinergic synapses, resulting in dose-limiting central and peripheral adverse effects. The most prominent adverse effects of AChE inhibitors are mediated by activation of peripheral M_2 and M_3 mAChRs and include bradycardia, gastrointestinal distress, excessive salivation, and sweating (Bymaster et al., 2002; Langmead et al., 2008; Wess et al., 2007). To better understand the role of each receptor subtype in the potential adverse side effects, and more importantly the reported efficacy, a more refined characterization of regional localization has been undertaken. Neuroanatomical studies using subtype-selective antibodies for the different mAChRs have established distinct patterns of expression for the different mAChR subtypes within the CNS (Levey et al., 1994; Levey et al., 1995; Levey et al., 1995; Levey et al., 1991). Additionally, the recent development and characterization of knockout (KO) mice for each of the mAChR subtypes has proven invaluable in understanding the central and peripheral functions of each subtype (Wess et al., 2007).

The M_1 mAChR is the predominant subtype expressed in the CNS, and is most densely located in the striatum, postsynaptically on the cell bodies of dendrites of hippocampal pyramidal neurons and granule cells, and in all layers of the cortex (Levey et al., 1995; Levey et al., 1991; Marino et al., 1998; Rouse et al., 1998). Importantly, M_1 mAChR activation has been shown to potentiate NMDA currents in the hippocampus and forebrain of rodents (Marino et al., 1998), and it is postulated that selectively activating M_1 mAChRs may alleviate some of the positive symptoms and cognitive deficits observed in schizophrenia through this mechanism (Marino et al., 1998; Tsai & Coyle, 2002). In addition, recent studies have shown that activation of M_1 mAChRs in wildtype animals increases the synaptic excitation of pyramidal cells in the medial PFC (mPFC), resulting in enhanced performance in mPFC-mediated behavioral paradigms, specifically the attentional set shift task (Shirey et al., 2009). Conversely, M_1-KO mice exhibited deficits in hippocampal and mPFC-mediated behavioral tasks, as observed in consolidation of contextual conditioned freezing and in radial arm maze, respectively (Anagnostaras et al., 2003). M_1-KO mice also have significant increases in spontaneous locomotor activity and enhanced amphetamine-induced hyperactivity associated with a twofold increase in DA efflux in the striatum, suggesting a role for M_1 mAChRs in modulating the dopaminergic system (Gerber et al., 2001; Miyakawa et al., 2001).

The M_4 mAChRs are widely distributed across several brain regions, including the cortex and hippocampus, with the densest expression occurring in the striatum (Hersch & Levey, 1995; Levey et al., 1991; Rouse et al., 1998). M_4 mAChRs can be expressed presynaptically, where they function as autoreceptors on striatal cholinergic interneurons or in midbrain projection neurons (Tzavara et al., 2004; Zhang et al., 2002),

and postsynaptically as modulatory receptors in the striatum, cortex, and hippocampus (Levey et al., 1991; Zang & Creese, 1997). M_4-KO mice show significantly higher basal locomotor activity and increased psychostimulant-induced DA efflux in the nucleus accumbens (Gomeza et al., 1999; Tzavara et al., 2004). Interestingly, targeted KO of M_4 in DA D1 receptors results in a similar psychostimulant response on DA efflux, suggesting a crucial role for M_4 in modulating dopaminergic circuitry related to schizophrenia (Jeon et al., 2010).

The M_1 and M_4 mAChRs emerged as potential targets for treating some of the symptoms associated with Alzheimer's disease and schizophrenia following the initial characterization of the M_1/M_4-preferring orthosteric agonist, xanomeline. In clinical studies, xanomeline produced a dose-dependent reduction in Alzheimer's disease-related behavioral disturbances, including vocal outbursts, suspiciousness, delusions, agitation, and hallucinations (Bodick et al., 1997a; Bodick et al., 1997b). Interestingly, xanomeline also improved blunted affect, something that few clinically available medications achieve. These aspects of Alzheimer's disease share remarkable similarities to those observed in schizophrenia, and the surprising efficacy observed raised the possibility that xanomeline might provide a novel approach for the treatment of schizophrenia. For these reasons, a subsequent 4-week, double-blind, placebo-controlled outcome trial was performed in subjects with schizophrenia (n=20) to evaluate the potential antipsychotic efficacy of xanomeline. In these studies, xanomeline treatment resulted in marked improvements in patients with schizophrenia when compared with the placebo group, as measured by the Brief Psychiatric Rating Scale (BPRS), Positive and Negative Syndrome Scale (PANSS), and Clinical Global Impression (Shekhar et al., 2008). Unlike tradition

antipsychotic medications, xanomeline showed efficacy in improving multiple domains of cognition, including visuospatial learning, verbal learning, attention/vigilance, speed of processing, and working memory, indicating efficacy in some aspects of cognition (Shekhar et al., 2008). Also of importance, xanomeline elicited its effects on the BPRS within one week of initiation, and continued improvement in the BPRS and PANSS were observed for the duration of the study.

In preclinical studies predictive of antipsychotic-like activity, xanomeline recapitulated many of the effects observed with atypical antipsychotics such as clozapine (Jones et al., 2005; Perry et al., 2001). Specifically, pretreatment with xanomeline reversed apomorphine and scopolamine-induced deficits in prepulse inhibition (PPI) of the acoustic startle reflex, a model of sensory information processing deficits, and increased extracellular levels of dopamine and c-fos expression in prefrontal cortex and nucleus accumbens, respectively. Xanomeline also attenuated amphetamine-induced increases in locomotor activity and increased dopamine turnover in the prefrontal cortex of rats, with similar efficacy to that observed in the clozapine and haloperidol control groups (Stanhope et al., 2001). This novel mechanism for modulating psychotic symptoms was further supported by studies reporting decreases in DA cell firing in the VTA, inhibition of conditioned avoidance responding, and reductions in apomorphine-induced climbing. Importantly, xanomeline did not induce catalepsy like other typical antipsychotics such as haloperidol, and the beneficial effects could be blocked by administration of the muscarinic receptor antagonist scopolamine (Shannon et al., 2000). In non-human primates, xanomeline produced functional dopamine antagonism and did not induce extrapyramidal side effects, as demonstrated by blockade of amphetamine and

apomorphine-induced motor unrest and stereotypies, mimicking observations in rodent models (Andersen et al., 2003). These findings demonstrate that xanomeline has a pharmacologic profile similar to currently available typical and atypical antipsychotics, indicating xanomeline could provide a novel approach for the treatment of psychotic symptoms in patients with Alzheimer's disease and schizophrenia.

Unfortunately, adverse effects were prevalent in patients who participated in the initial clinical trials with xanomeline, as well as in preclinical animal models receiving elevated doses of xanomeline (Andersen et al., 2003; Bodick et al., 1997a). These included gastrointestinal cramping, salivation, and vomiting, and were thought to be due to the nonselective nature of xanomeline, which resulted in activation of peripheral M_2 and M_3 mAChRs. Although these side effects did not lead to any mandatory discontinuation, there was voluntary attrition and these dose limitations prevented xanomeline from long-term clinical use. These studies, however, provided important clinical validation of the potential of mAChR agonists as novel therapeutic agents for treatment of psychosis as well as cognitive disturbances in schizophrenia patients. These findings also raised the question of whether activation of M_1, M_4, or both receptor subtypes is critical for the observed clinical effects, and highlight the need for the development of selective ligands to address this question.

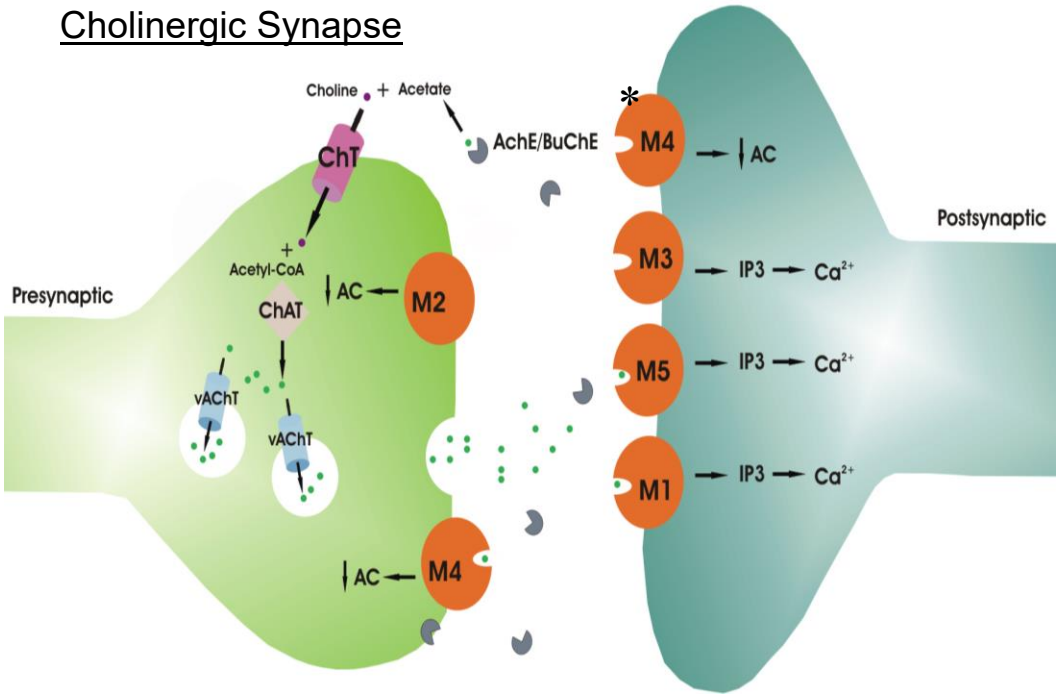


Figure 6. Synaptic Localization of M_1-M_5 mAChR. M_1, M_3, and M_5 are found exclusively on the postsynaptic neuron. M_4 is found on both pre and postsynaptic neurons and M_2 is exclusively found on presynaptic neurons. Figure adapted from (Jones et al., 2012).

Allosteric mAChR modulators

Over the last decade, a novel approach has been undertaken in the discovery of mAChR ligands that selectively activate a particular receptor subtype by actions at sites that are topographically distinct and less highly conserved than the orthosteric binding site of ACh, termed allosteric sites (Conn et al., 2009). The use of allosteric activators has been well validated during the development of benzodiazepines, which are allosteric activators of $GABA_A$ receptors and provide a safe, effective treatment approach for anxiety disorders without inducing adverse effects of direct-acting $GABA_A$ receptor agonists (Ehlert et al., 1983). Allosteric activators of mAChRs possess high subtype selectivity and can show different modes of action. Allosteric agonists can activate the receptor subtype directly and do not require the presence of ACh. Alternatively, positive allosteric modulators (PAMs) do not directly activate the receptor, but bind to an allosteric site distinct from the ACh-binding site and potentiate the effects of endogenous ACh. One potential advantage of the use of mAChR PAMs is that these ligands have no intrinsic activity and can only exert their effects in the presence of ACh at a given synapse, thereby maintaining some level of activity dependence of endogenous receptor activation. To date, these novel agonists and allosteric activators of the different mAChR subtypes have shown robust efficacy in preclinical models of antipsychotic-like activity and/or enhancement of cognitive function, and possess suitable physiochemical properties for optimization as potential clinical candidates.

Development of selective mAChR ligands

Sizeable advances have been made in the development of potent and selective allosteric modulators of mAChRs by our group at the Vanderbilt Center for Neuroscience

Drug Discovery as well as in others that allow for further investigation into the role of M_1 and M_4 in the preclinical and clinical efficacy observed with xanomeline (Bridges et al., 2010; Conn et al., 2009). Recently, several systemically active, M_1 allosteric agonists and PAMs are proving to be useful tools for the study of selective activation of M_1 mAChRs *in vitro* and in animal models predictive of antipsychotic-like activity and enhancement of cognition. To date, several M_1 allosteric agonists have been described, including TBPB, 77-LH-28-1, AC260584, VU0186470 and VU0357017. TBPB is a potent (EC_{50}=280 nM) and selective (>30 μM vs M_2–M_5) M_1 allosteric agonist in cell-based systems (Jones et al., 2008). M_1 mAChR activation by TBPB potentiated NMDAR currents in CA1 hippocampal pyramidal cells, a physiological response that is linked to the facilitation of learning and memory. Functional anatomical studies with TBPB revealed a Fos expression pattern that was consistent with what is observed with the atypical antipsychotic clozapine, with increased numbers of Fos-containing cells in the mPFC and nucleus accumbens but not the dorsal striatum. Additionally, TBPB reversed amphetamine-induced hyperlocomotion in a dose dependent manner within a dose range that did not induce side effects associated with the non-selective stimulation of peripheral mAChRs, as measured using a modified Irwin test battery or catalepsy, preclinical measures of extrapyramidal motor symptoms (Jones et al., 2008).

Studies describing the discovery of BQCA (benzyl quinolone carboxylic acid) represented a breakthrough in the development of selective M1 PAMs. BQCA does not bind at the orthosteric ACh-binding site, but increases the affinity of the M_1 mAChR for ACh, as represented by a 129-fold leftward shift of the ACh concentration response curve (Ma et al., 2009). In *ex vivo* slice electrophysiology studies, BQCA increased inward

currents in mPFC pyramidal cells and spontaneous excitatory postsynaptic currents in response to the non-selective mAChR agonist carbachol, and these effects were not observed in M_1-KO mice (Shirey et al., 2009). BQCA also has pharmacokinetic properties that make it suitable for *in vivo* experiments. As such, behavioral studies revealed efficacy in a mPFC-dependent discrimination reversal learning task in a transgenic mouse model of Alzheimer's disease (Shirey et al., 2009). In addition, reports indicate efficacy in reversing scopolamine-induced deficits in the acquisition of contextual fear conditioning, improved novel object recognition, improving cognition on a top-down processing task, and altered sleep architecture by enhancing wakefulness and decreasing delta sleep (Chambon et al., 2012; Gould et al., 2015; Ma et al., 2009). Similar to the effects observed with TBPB, xanomeline, and clozapine, BQCA increased Fos expression in the forebrain and dose dependently reversed amphetamine-induced hyper-locomotion in mice (Ma et al., 2009).

A more recently discovered selective M_1 PAM, VU0453595, has been evaluated in models of NMDA receptor hypofunction in mice (Ghoshal et al., 2016). In these studies, VU0453595 bath application potentiated M_1-mediated muscarinic long term depression (LTD) in the prefrontal cortex, a response that is dependent on M_1 activation. Interestingly, repeated PCP treatment in mice during adolescence resulted in a deficit in muscarinic LTD; however, bath application of VU0453595 reversed this response. When assessed *in vivo*, VU0453595 reversed chronic PCP-induced deficits in performance in PFC-dependent behavioral assays, suggesting a role for M_1 mAChRs in the cognitive deficits and negative symptoms associated with schizophrenia (Ghoshal et al., 2016).

Collectively, these data demonstrate that selective activation of M_1 by both M_1 allosteric agonists and PAMs is sufficient to reproduce some of the effects of xanomeline and clinically available antipsychotics in animal models that are predictive of antipsychotic-like activity and cognitive enhancement. These studies also support the idea that M_1 activation may play a critical role in hippocampal- and mPFC-dependent cognitive functions and suggest that M_1 allosteric activators may serve as an important approach for the treatment of the prefrontal cortical deficits observed in patients with schizophrenia.

To investigate the relationship between M_1 PAM potency and behavioral efficacy, tool compounds with nanomolar potency that retain suitable physiochemical properties for *in vivo* dosing need to be developed. To address this, the M_1 PAM tool compound, VU6004256, has been reported. VU6004256 is a potent potentiator of mouse M_1 (EC_{50} = 155 nM, 88% ACh Max) and is ~20-fold more potent than VU0453595. In addition, VU6004256 displays mouse brain:plasma partitioning (K_p) coefficient of 4.84 and an unbound brain:plasma coefficient ($K_{p,uu)}$ of 2.6. In addition to selectivity for M_1 ($M_{2\text{-}5}$ EC_{50}s >30 μM), VU6004256 also displayed no off-target pharmacology at over 68 GPCRs, ion channels and transporters in an ancillary pharmacology radioligand binding panel (% inhibition @ 10 μM <50%). Therefore, VU6004256 represents a more potent and centrally penetrant M_1 PAM compared to VU0453595 to explore efficacy in behavioral models of schizophrenia (Chapter V).

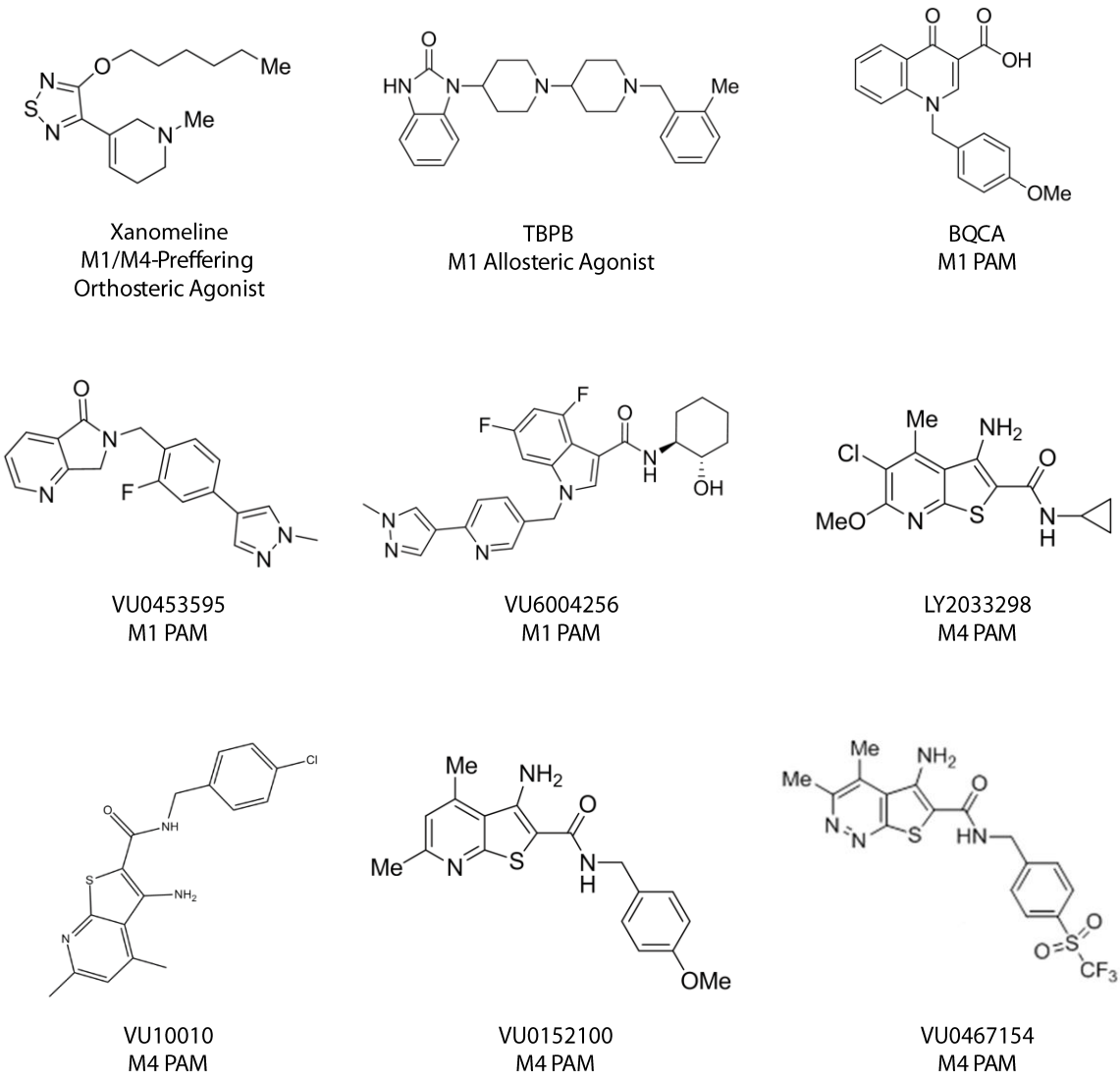


Figure 7. Summary of previously described M_1 and M_4 orthosteric and allosteric activators.

There have also been breakthroughs in the last decade in the development of M_4-selective ligands, as represented by LY2033298, VU0010010, VU0152100, and VU0467154. One of the first M_4 PAMs described was LY2033298, a highly selective compound with suitable properties for *in vivo* dosing and no detectable activity at any of the other mAChR subtypes (Chan et al., 2008). Interestingly, LY2033298 had no effects in preclinical studies when administered alone, but was able to potentiate the effects of a sub-threshold dose of the non-selective mAChR agonist oxotremorine to reverse dopamine receptor agonist-induced disruptions in prepulse inhibition and the inhibition of conditioned avoidance responding (Chan et al., 2008; Leach et al., 2010). In addition, neurochemical studies using *in vivo* microdialysis revealed LY2033298 potentiated oxotremorine-stimulated DA release in the PFC but not nucleus accumbens, and had no measurable effect when administered alone (Chan et al., 2008). It was hypothesized that species differences resulting in decreased potency at rat M_4 vs. human M_4 receptors was responsible for a lack of in vivo efficacy when administered alone, highlighting the need for more potent tool compounds to more accurately predict efficacy in the clinic. Nonetheless, in combination with an ACh agonist, LY2033298 demonstrated efficacy in animal models predictive of antipsychotic-like activity and provided support for further development of other selective M_4 PAMs.

Following closely on the heels of the reports of LY2033298, Shirey et al. (Shirey et al., 2008) reported the chemical and pharmacological characterization of the M_4 PAM VU10010, which has an EC_{50} of 400 nM for potentiation of ACh effects at the rat M_4 and a 47-fold leftward shift in the functional ACh response curve (>30 μM vs M_1–M_3, M_5). Interestingly, VU10010 was shown to be an allosteric potentiator that increased both the

efficiency of the coupling of the M_4 mAChR to G-proteins and the affinity of the M_4 mAChR for ACh (Shirey et al., 2008). Unfortunately, VU10010 had unsuitable physiochemical properties for *in vivo* studies. Further optimization of this chemical series resulted in the discovery of VU0152099 and VU0152100. Both of these M_4 PAMs retained high mAChR subtype selectivity for M_4 relative to the other mAChRs (>30 μM vs M_1–M_3, and M_5) and further functional selectivity in a screen of 15 other GPCRs that are highly expressed in the brain (Brady et al., 2008). These novel M_4 PAMs are highly centrally penetrant, unlike VU10010, with pharmacokinetic properties ideal for studies in animal models of psychosis and cognition. Consequently, behavioral studies utilizing these compounds revealed efficacy in a preclinical model predictive of antipsychotic-like activity as represented by a robust reversal of amphetamine-induced hyper-locomotion (Brady et al., 2008). VU0152100 also reversed amphetamine-induced disruptions in the acquisition of contextual fear conditioning in rats, suggesting behavioral efficacy in preclinical models used to assess cognition (Byun et al., 2014). Interestingly, these more optimized compounds exhibited effects *in vivo* when administered alone, suggesting there is sufficient ACh present in the synapse for an M_4 PAM to elicit a pharmacologically relevant responses under normal conditions.

To date, the most potent M_4 PAM described is VU0467154 (5-amino-3,4-dimethyl-*N*-(4-((trifluoromethyl)sulfonyl)benzyl)thieno[2,3-*c*]pyridazine-6-carboxamide). VU0467154 is a potent potentiator of rat M_4 (EC_{50} = 17.7 nM, 68% ACh Max), shows no activity at any of the other mAChRs at concentrations greater than 30 μM, and has little to no ancillary off-target pharmacology when assessed in a Ricerca lead profiling screen (Bubser et al., 2014). VU0467154 also exhibits a favorable

pharmacokinetic profile for *in vivo* studies in rodents ($t_{1/2}$ = 5.7 h; CL_p = 7.8 mL/min/kg; F = 61%; $K_{p,uu}$ =0 .41), though its utility is limited to that of a tool compound due to differences in potency at human M_4 that preclude its development for use in clinical populations (human M_4 EC_{50} = 627 nM). In behavioral studies, VU0467154 dose-dependently reversed both MK-801- and amphetamine-induced increases in locomotor activity in wildtype, but not M_4 KO mice. In addition, dose-dependent reversals of MK-801-induced disruptions in a touch screen operant task were observed with VU0467154 co-administration to wildtype, but not M_4 KO mice. VU0467154 also demonstrated efficacy in reversing MK-801-induced disruptions in acquisition of contextual fear conditioning, but perhaps more interesting was the enhancement of acquisition of this task in wildtype animals when VU0467154 was administered alone (Bubser et al., 2014). Collectively, these studies indicate that the observed antipsychotic-like and cognition enhancing activity of VU0467154 are mediated through an M_4-dependent mechanism

Using polysomnography and quantitative electroencephalographic (EEG) recordings, VU0467154 was shown to induce state-dependent alterations in sleep architecture and arousal in a manner similar to atypical antipsychotics, including delayed Rapid Eye Movement (REM) sleep onset, increased cumulative duration of total and Non-Rapid Eye Movement (NREM) sleep, and increased arousal during waking periods (Gould et al., 2016). Additionally, in acute MK-801 models of NMDAR hypofunction, both VU0467154 and clozapine reduced elevations in high frequency gamma power, consistent with and antipsychotic-like mechanism of action (Gould et al., 2016; Hiyoshi et al., 2014). In summary, selective positive allosteric modulation of M_4 muscarinic receptors by VU0467154 reverses the behavioral, cognitive, and sleep deficits in an acute

model of NMDAR hypofunction, which provides strong evidence for the broader therapeutic utility for M_4 PAMs in the treatment of complex symptoms observed in patients with schizophrenia.

Outline of current studies

Thus, in Chapter II, we performed studies to assess the ability of selective M_4 PAMs to modulate mesolimbic circuitry and behaviors that are predictive of antipsychotic-like activity. Specifically, we examined the effects of two novel M_4 PAMs, VU0152100 and VU0467154, to reverse amphetamine-induced hyperlocomotor activity. Next, we evaluated changes in cerebral blood volume (CBV) in rats in specific regions of interest following dosing with amphetamine, or a combination of amphetamine and the M_4 PAM VU0152100. We then assessed changes in amphetamine-evoked dopamine efflux in the nucleus accumbens (NAS) and caudate putamen (CP), and how co-administration of VU0152100 affected this response. In these studies, we hypothesized that the antipsychotic-like efficacy observed with M_4 PAMs could be due to modulation of mesolimbic dopamine release.

In Chapter III, we performed studies to elucidate the role of M_4 mAChRs in cortical circuits thought to be disrupted in schizophrenia. To achieve this, whole-cell patch-clamp experiments were utilized to measure changes in hallucinogen-evoked changes in excitatory signal in the PFC, and the ability of the M_4 PAM, VU0467154, to mediate this response. To determine if M_4 mAChRs could be exerting this effect through modulation of thalamocortical circuitry, an optogenetic whole-cell electrophysiology study was carried out. To further support these findings, in situ hybridization studies were

performed to confirm M_4 mAChR expression on thalamocortical neurons. Next, we determined the effect of pretreatment with an M_4 PAM on hallucinogen-induced head-twitch response in mice, a behavior known to be regulated by thalamocortical glutamate release. Then, we measured performance in an operant-based spatial alternation task, which is thought to require thalamocortical signaling, following dosing with an NMDA receptor antagonist in the presence or absence of an M_4 PAM. We hypothesized that deficits in glutamatergic signaling in the PFC induced by NMDA receptor antagonists or hallucinogens could be normalized by M_4 PAMs. Additionally, we postulated that the actions of M_4 PAMs could be working through modulation of thalamocortical circuitry, and regulation of this circuit could, in part, result in improvements in PFC-mediated behavior. Finally, we assessed whether M_4 potentiation would result in reversal of electrophysiological deficits in a genetic model of NMDAR hypofunction.

In Chapter IV, we examined the effects of repeated dosing with the M_4 PAM VU0467154 on learning a touchscreen-based visual pairwise discrimination task. To determine if the effects of M_4 PAM administration were due to acquisition or consolidation of new learning, studies were carried out in which animals were treated with an M_4 PAM before or directly following training in the discrimination task. To ensure repeated dosing with an M_4 PAM would not lead to tolerance of the antipsychotic-like or cognitive enhancing effects, or changes in pharmacokinetic disposition, behavioral studies were performed followed by tissue collection. We hypothesized that repeated dosing with an M_4 PAM would lead to cognitive enhancement, at least in part through actions during memory consolidation during sleep, and that the antipsychotic-like profile would remain intact following this repeated dosing paradigm.

In Chapter V, we sought to examine the role of M_1 PAMs in modulating PFC-mediated cognition in a mouse model of NMDA receptor hypofunction. To achieve this, we observed changes in muscarinic-long term depression (LTD) in the PFC of NR1 deficient mice, followed by the effects of the M_1 PAM VU6004256. Next, we evaluated changes in layer V PFC pyramidal cell firing rate in NR1 KD animals before and after M_1 PAM administration. To determine if M_1 PAMs would be efficacious in behavior models reliant on PFC signaling, studies were conducted in WT and NR1 KD animals evaluating performance in novel object recognition and cue-mediated conditioned freezing behavior. Finally, the effects of two M_1 PAMs, VU6004256 and VU0453595, were observed in spontaneous locomotor behavior in WT and NR1 KD mice. We hypothesized that M_1 PAM administration would normalize electrophysiological deficits that are related to abnormal behavior in a genetic animal model of NMDA receptor hypofunction.

CHAPTER II

SELECTIVE POTENTIATION OF M_4 MUSCARINIC ACETYLCHOLINE RECEPTORS MODULATES MESOLIMBIC DOPAMINERGIC CIRCUITRY IN MODELS PREDICTIVE OF ANTIPSYCHOTIC-LIKE ACTIVITY

Introduction

Patients diagnosed with schizophrenia suffer from a host of debilitating symptoms, including positive symptoms, negative symptoms, and deficits in cognition (American Psychiatric Association, 2013; Nuechterlein et al., 2004). Unfortunately, all clinically available typical and atypical antipsychotics are primarily efficacious in treating the positive symptoms, with little effect on the negative symptoms or cognitive deficits (Swartz et al., 2008). Current antipsychotic medications are also plagued with only partial patient responsiveness, treatment resistance, delayed onset of action, and a long list of dose-limiting side effects, including extrapyramidal motor effects and metabolic disorders (Bak et al., 2014; Lieberman et al., 2003; Parsons et al., 2009). As a result, there remains a large unmet medical need to develop novel treatment strategies that address all three symptoms domains without the complexities present with current antipsychotic medications.

As discussed in Chapter I, there is growing evidence to suggest selective activators or potentiators of mAChRs may present a new treatment approach for the treatment of the psychotic symptoms and cognitive deficits observed in schizophrenia and Alzheimer's disease patients. Much of this interested stemmed from preclinical and

clinical findings with the M_1/M_4-preferring mAChR orthosteric agonist, xanomeline. In a large multicenter clinical trial, xanomeline produced modest, although statistically insignificant, effects on cognitive function; however, strikingly robust efficacy was observed in treating the psychotic symptoms in Alzheimer's disease patients (Bodick et al., 1997a; Bodick et al., 1997b). In particular, xanomeline induced dose-dependent reductions in suspiciousness, delusions, hallucinations, and other behavioral disturbances and psychotic symptoms that are highly analogous to those observed in schizophrenia. Based on these initial findings, a double blind, placebo-controlled clinical study was performed to determine if xanomeline also had antipsychotic efficacy in patients with schizophrenia (Shekhar et al., 2008). Interestingly, xanomeline produced robust improvements in both positive and negative symptoms in this population and also improved cognitive performance, particularly in measures of verbal learning and short-term memory. While xanomeline ultimately failed to progress in the clinic due to off target activation of peripherally expressed M2 and M3 receptors, it did shine light on the potential of muscarinic system modulation for the treatment of several symptom domains in schizophrenia.

One potential mechanism for the efficacy observed in treating the positive symptoms may be through modulation of mesolimbic circuitry. In support of this hypothesis, anatomical studies in rodents indicate that M_4 mAChRs are abundantly expressed in several key regions of the mesocorticolimbic dopaminergic circuitry, including the VTA, NAS and mPFC. In particular, M_4 mAChRs are reported to be localized on both cholinergic interneurons and medium spiny GABAergic neurons within the NAS and dorsal striatum (Hersch & Levey, 1995; Zhang et al., 2002), presynaptically

on glutamatergic terminals in multiple cortical regions and presynaptically on cholinergic terminals from midbrain CH5/6 cholinergic projections providing monosynpatic activation of dopamine neurons in the VTA (Garzon & Pickel, 2000; Levey, 1993; Levey, 1996; Oakman et al., 1999; Sugaya et al., 1997; Volpicelli & Levey, 2004; Westerink et al., 1998; Westerink et al., 1996). In line with this expression pattern, M_4 KO mice show elevated extracellular levels of dopamine in the NAS compared with wildtype controls (Tzavara et al., 2004). Moreover, M_4 KO mice also show increased sensitivity to psychostimulants which result in elevated levels of extracellular dopamine release in the NAS. While the direct mechanism responsible for this change is unclear, evidence indicates that one important site of regulation may be the M_4 inhibitory autoreceptor feedback on cholinergic terminals in the VTA. Consistent with this hypothesis, loss of M_4 mAChR control on Ch5/Ch6 cholinergic projections results in increased extracellular levels of ACh in the VTA in M_4 KO mice, leading to enhanced activation of midbrain dopamine neurons (Tzavara et al., 2004). This disinhibition of the mesolimbic dopamine neurotransmission observed in M_4 KO mice also provides a likely mechanism for the exacerbated behavioral response to psychostimulants in these animals (Gomeza et al., 1999; Gomeza et al., 2001). Taken together, these findings indicate that the absence of M_4 mAChR function in the M_4 KO mice results in a state of hyperexcitability of the mesolimbic dopaminergic circuitry, a state thought to underlying symptoms associated with schizophrenia, and suggest that activation of M_4 mAChRs may reduce mesolimbic and mesostriatal dopaminergic neurotransmission.

Thus, using behavioral, neurochemical, and imaging approaches, the following studies were performed to assess the ability of selective M_4 PAMs to modulate

dopaminergic circuitry involved in schizophrenia. To complete these studies, two distinct M_4 PAMs were used, VU0467154 and VU0152100. As discussed in Chapter I, VU0152100 represented the first highly selective, potent M_4 PAM with pharmacokinetic properties suitable for *in vivo studies* (Byun et al., 2014). VU0467154 is a closely related analog with more optimized potency and pharmacokinetic properties that allows for insight into the role of M_4 receptors in modulating mesolimbic circuitry, as well as in more complex electrophysiological and behavioral paradigms (Chapters III and IV).

Methods

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.

Chemicals and Biological Materials

Chemicals for the synthesis of VU0467154 and VU0152100 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.

Drugs

D-Amphetamine hemisulfate was obtained from Sigma-Aldrich. VU0467154 and VU0152100 were 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. Amphetamine was dissolved in sterile water. In rats, amphetamine was administered subcutaneously (SC) at 1 mL/kg. In mice, drugs were administered in a volume of 10 mL/kg.

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 (Jones et al., 2008). 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. The following paradigm was used to assess drug effects on amphetamine-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 or amphetamine 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).

In Vivo Microdialysis and Locomotor Activity

Guide cannulae (BioAnalytical Systems, West Lafayette, IN) were implanted into the NAS (AP +1.5 mm bregma, ML −1.5 mm, DV −7.8 mm) or CP (AP 1.0 mm, ML −2.5 mm, DV −5.5 mm). After recovery for 4–7 days, microdialysis probes (2 mm membrane length) were inserted the night before the experiment using methods previously reported by (Perry et al., 2001). The following day, rats were placed into open field chambers as used for the amphetamine-induced hyperlocomotion studies. Probes were perfused with aCSF (150 mM NaCl, 3 mM KCl, 1.7 mM CaCl2, and 0.9 mM MgCl2) and dialysate samples were collected in 15-min fractions onto a 96-well plate using a refrigerated fraction collector (EFC-82, Eicom). Following a 2-h equilibration, four baseline samples were collected, followed by a 30-min pre-treatment with either vehicle or VU0152100 (56.6 mg/kg, i.p.). Then all rats were injected with either vehicle or amphetamine (1 mg/kg, s.c.) and dialysate samples were collected for another 120 min. At the end of the study, probes were perfused with methyl blue to verify placement. Dialysates were analyzed using an Eicom ECD-700 HPLC system. Dopamine and its two major metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were separated at a flow rate of 400 µl/min using a mobile phase consisting of 0.1 M citrate-acetate buffer/methanol (80 : 20), 220 mg/l sodium octanesulfonic acid, and

5 mg/l EDTA and quantitated by electrochemical detection. Chromatograms were analyzed using PowerChrom (eDAQ, Denistone East, NSW, Australia). Only animals with accurate probe placement that showed three consecutive stable baseline values (within ⩽20%) were included in the statistical analysis.

Pharmacologic Magnetic Resonance Imaging (phMRI)

Isoflurane-anesthetized rats, with preimplanted jugular catheters, underwent endotracheal intubation (14 G catheter), insertion of i.p. catheters (size P50, Braintree Scientific, Braintree, MA), and mechanical ventilation (Kent Scientific, Litchfield, CT; O2:N2O 1 : 2; 2% isoflurane). For scanning, isoflurane was set to 0.88% and pancuronium bromide administered (1 mg/kg, i.p.; BoundTree Medical, Dublin, OH). Heart rate, respiration, and rectal temperature were continuously measured and temperature maintained through a thermocoupled heating unit (SAM-PC; SA Instruments, Encinitas, CA). End-tidal CO2 was continuously monitored (Invivo Research, Orlando, FL). PhMRI data were acquired with a Doty Litz 38-mm transmit-receive radio frequency coil using a 9.4 T Varian magnet controlled by a Varian Inova console (Agilent, Palo Alto, CA). High-resolution fast spin-echo (fse) structural images were collected (repetition time (TR) 2550 ms; effective echo time (TEeff) 40 ms; number of excitations (NEX) 2; 128 × 128 matrix; 35 × 35 mm2 field of view; 11 contiguous slices, 1.5 mm thick). Pre-contrast reference images and post-contrast functional images were acquired (fse: TR 2600 ms; TEeff 36 ms; NEX 2; 64 × 64 matrix). To measure cerebral blood volume (CBV), Molday iron oxide nanoparticles (30 nm; 20 mg/kg, i.v.; BioPAL, Worcester, MA) were injected. For amphetamine interaction experiments, rats

were pre-treated for 15 min with vehicle or 56.6 mg/kg VU0152100 (i.p.), then a 15-min baseline was collected, after which rats were administered with vehicle or a 1 mg/kg amphetamine (i.p.) followed by 45 min of continuous acquisition. In a separate study to assess the effects of VU0152100 alone on fractional CBV changes, an initial 15-min baseline was collected, followed by administration of vehicle or VU0152100 (56.6 mg/kg, i.p.), and 45 min of continuous image acquisition.

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

Functional Connectivity Analysis

The strength of functional connections between brain regions was quantified by computing the Pearson linear correlation coefficient between the integral of the CBV response to amphetamine for each ROI pair for the vehicle/amphetamine and

VU0152100/amphetamine groups (Schwarz et al, 2007b). Correlation coefficients, r, were converted to z-scores using Fisher's transformation (z=ln[(1+r)/(1−r)]/[2*(1/(N−3))0.5]) and thresholded at |Z|>2 (in-house MATLAB code). Permutation analysis (Holmes et al, 1996) was used to identify the correlations that were significantly different (Δz, p<0.05) between vehicle/amphetamine and VU0152100/amphetamine groups (in-house MATLAB code).

Results

VU0467154 Reverses Amphetamine-Induced Hyperlocomotion in Rats after IP and PO Dosing

The M_1/M_4-preferring agonist xanomeline has previously been reported to have an APD-like profile, including reversal of amphetamine-induced hyperlocomotion (Stanhope et al., 2001). 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 8). Amphetamine produced a dose- and time-dependent increase in locomotor activity ($F_{dose(5,1008)} = 96.37$, $p < 0.001$; $F_{time(23,1008)} = 58.99$, $p < 0.001$; and $F_{dose\times time(115,1008)} = 6.64$,$p < 0.001$), significant at doses of 0.3–1 mg/kg ($F_{5,47} = 29.47$, $p < 0.001$). VU0467154 produced a robust dose-dependent reversal of amphetamine-induced hyperlocomotion after IP ($F_{dose(6,1032)} = 40.97$, $p < 0.001$; $F_{time(23,1032)} = 42.78$, $p < 0.001$; and $F_{dose\times time(138,1032)} = 2.66$, $p < 0.001$) and PO ($F_{dose(6,1176)} = 45.54$, $p < 0.001$; $F_{time(23,1176)} =$

65.09, $p < 0.001$; and $F_{dose \times time(138,1176)} = 2.55$, $p < 0.001$) routes of administration (Figure 9). 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 ($F_{6,55} = 11.10$, $p < 0.001$).

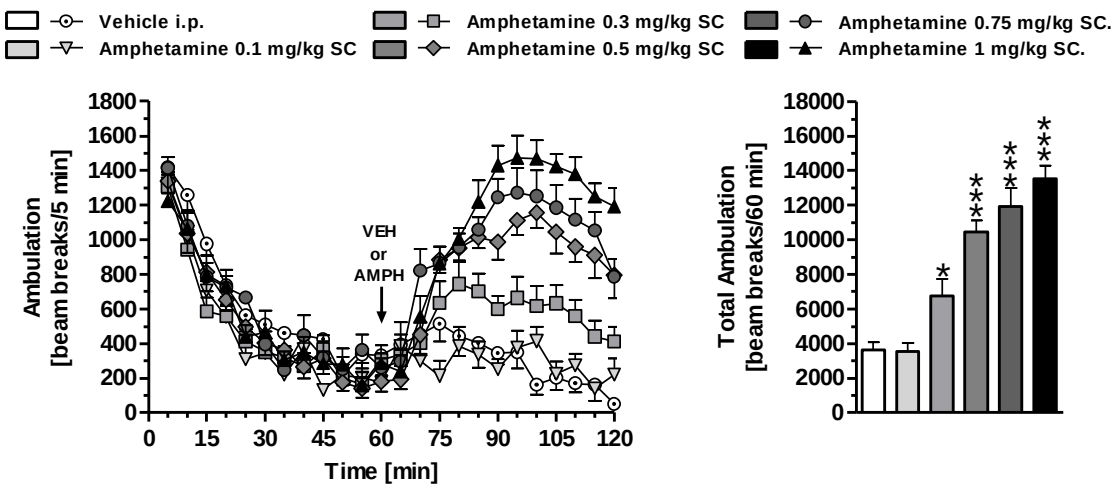


Figure 8. Amphetamine (AMPH) dose-dependently increases open field locomotor activity. The time course of locomotor activity is shown on the left and total locomotor activity during the 60 min period following amphetamine administration on the right. Data are means ± SEM of 6–8 animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle (ANOVA followed by Dunnett's test).

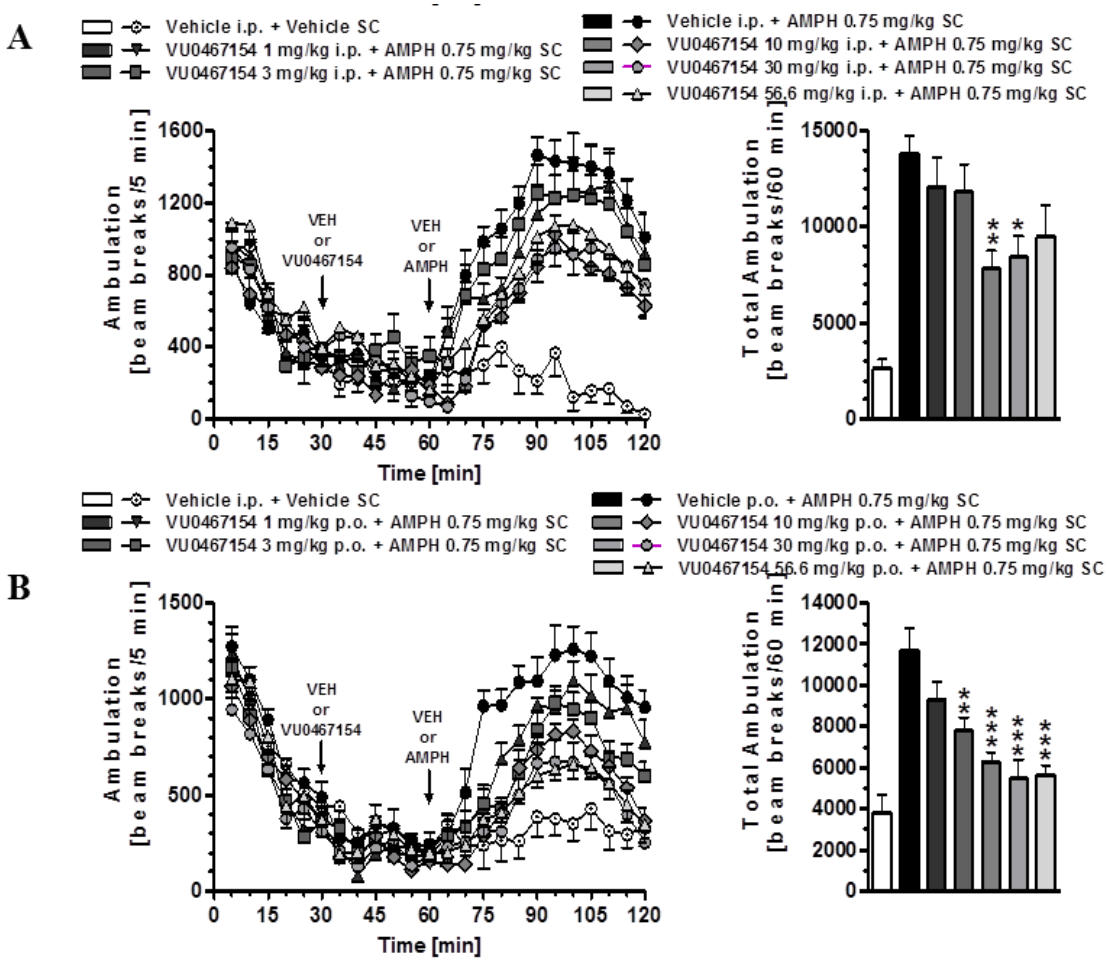


Figure 9. VU0467154 reverses amphetamine-induced hyperlocomotion in rats. Systemic (IP [A] or PO [B]) administration of VU0467154 dose-dependently reverses amphetamine-induced hyperlocomotion. The time course of locomotor activity is shown on the left and total locomotor activity during the 60 min period following amphetamine administration on the right. Data are means ± 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).

VU0467154 Reverses Amphetamine-Induced Hyperlocomotion in Wild-type but Not in M_4 KO Mice

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 10). Amphetamine produced a dose- and time-dependent increase in locomotor activity in both wild-type and M_4 KO mice ($F_{dose(7,3696)} = 337.8$, $p < 0.001$; $F_{time(47,3696)} = 31.77$, $p < 0.001$; and $F_{dose\times time(329,3696)} = 9.99$, $p < 0.001$). 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 ($F_{dose(3,77)} = 85.81$, $p < 0.001$; $F_{genotype(1,77)} = 1.08$, ns; and $F_{dose\times genotype(3,77)} = 1.02$, ns; Figure 10). VU0467154 (0.3–30 mg/kg) produced a robust reversal of the amphetamine-induced hyperlocomotion in wild-type mice ($F_{dose(5.2550)} = 68.42$, $p < 0.001$;$F_{time(47,2550)} = 68.73$, $p < 0.001$; and $F_{dose\times time(235,2555)} = 2.91$, $p < 0.001$), significant at doses of 3–30 mg/kg ($F_{5,59} = 9.13$, $p < 0.001$; Figure 11A). In contrast, VU0467154 had no effect on the time course of amphetamine-induced hyperlocomotion in M_4 KO mice ($F_{treatment(2,1296)} = 0.86$, ns; $F_{time(47,1296)} = 15.33$, $p < 0.001$; and $F_{dose\times time(94,1296)} = 0.47$, ns), nor did it reduce the total number of beam breaks compared with the animals treated with vehicle plus amphetamine ($F_{(2,29)} = 0.87$, ns; Figure 11B).

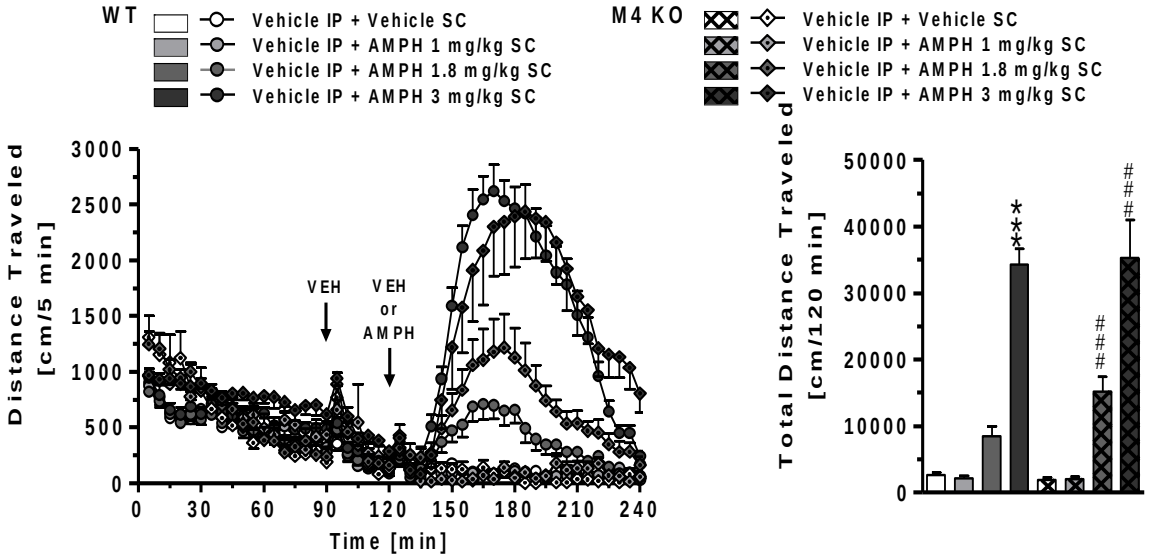


Figure 10. Amphetamine-induced hyperlocomotion in WT and M_4 KO mice. Amphetamine (AMPH) dose-dependently increases open field locomotor activity in wild-type (WT) and M_4 KO mice. The time course of locomotor activity is shown on the left and total locomotor activity during the 120 min period following amphetamine administration on the right. Data are means ± SEM of 9–13 wild-type mice and 10–11 M_4 KO mice per group. *** $p < 0.001$ vs wild-type vehicle + vehicle (A); $^{###}p < 0.001$ vs M_4 KO vehicle + vehicle (A); (ANOVA followed by Bonferroni's test).

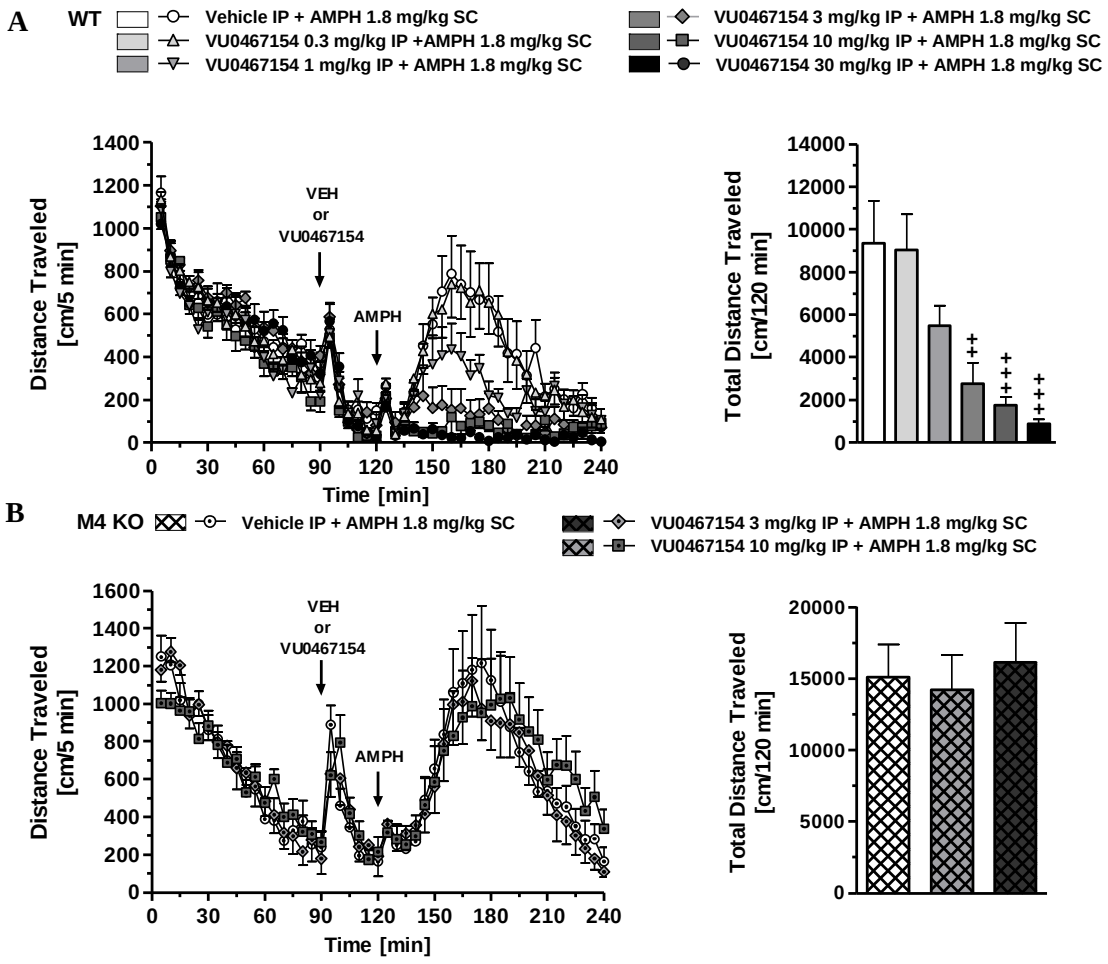


Figure 11. VU0467154 reverses amphetamine-induced hyperlocomotion in wild-type, but not M_4 KO mice. (A) In wild-type mice, administration of VU0467154 dose-dependently reverses amphetamine-induced hyperlocomotion. (B) VU0467154 does not reverse amphetamine-induced hyperlocomotion in M_4 KO mice. The time course of locomotor activity is shown on the left and total locomotor activity during the 120 min period following amphetamine administration on the right. Data are means ± SEM of 9–13 wild-type mice and 10–11 M_4 KO mice per group. $^{++}p < 0.01$, $^{+++}p < 0.001$ vs wild-type vehicle + amphetamine (ANOVA followed by Bonferroni's test).

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

In order to more comprehensively assess the specific brain regions underlying the effects of VU0152100 in reversing the amphetamine-induced changes in behavior, phMRI was used to examine the effects of a behaviorally relevant dose of VU0152100 (56.6 mg/kg, i.p.) on amphetamine-evoked (1 mg/kg, i.p.) alterations in CBV. As shown in the group-averaged CBV maps (Figure 12A) and time courses (Figure 12B), amphetamine robustly increased CBV in the NAS and CP, areas of high dopamine transporter expression, as well as in extrastriatal areas, including the thalamus, hippocampus, and retrosplenial cortex. Pre-treatment with VU0152100 (56.6 mg/kg, i.p.) significantly blunted amphetamine-evoked increases in CBV in the medial thalamus ($F_{2, 31}$=15.7, p<0.0001), hippocampus ($F_{2, 31}$=11.7, p<0.001), CP ($F_{2, 31}$=10.3, p<0.001), NAS ($F_{2, 31}$=9.31, p<0.001), and retrosplenial cortex ($F_{2, 31}$=3.8, p<0.05) (Figure 12B). There were no significant CBV changes in response to amphetamine alone or in combination with VU0152100 ($F_{2, 31}$=0.94, NS) in the PFC (Figure 12B); however, the lack of observed amphetamine effects may have been due in part to the anesthetized preparation and/or the exclusion of the two most anterior brain slices (1.5 mm in thickness) in the analysis to avoid partial volume effects as these slices also include the frontal pole and olfactory cortex, respectively. There were also no significant CBV responses detected in groups treated with vehicle/vehicle (Figure 12) or VU0152100 alone (data not shown).

Functional connectivity analysis of the above phMRI data revealed multiple inter-regional correlations in neural activation elicited by amphetamine in rats, while pre-

treatment with VU0152100 (56.6 mg/kg, i.p.) modified the amphetamine-induced correlation pattern; fewer significant inter-regional correlations were found in the group pre-treated with VU0152100 before amphetamine (data not shown). Permutation analysis identified significant differences between the two treatment groups (the statistical threshold was set at $p<0.05$ and reported as z-scores), including retrosplenium-hippocampus ($\Delta z=3.4499$), NAS-motor cortex ($\Delta z=3.3348$), retrosplenium-motor cortex ($\Delta z=3.2268$), and NAS-medial thalamus ($\Delta z=3.1553$) (Figure 12C). Other ROI pairs with significant differences included the NAS-CP ($\Delta z=3.0555$), motor cortex with hippocampus ($\Delta z=2.8753$), and visual cortex ($\Delta z=2.7884$); PFC-sensory cortex ($\Delta z=2.3990$); and substantia nigra-amygdala ($\Delta z=2.3409$).

The effects of VU0152100 on peripheral cardiovascular parameters, including blood pressure and heart rate, were assessed since changes might confound hemodynamic readouts of brain function (Ferrari et al., 2012). Compared with vehicle treatment, VU0152100 (56.6 mg/kg, i.p.) did not change arterial blood pressure ($F(\text{treatment})_{1, 72}=9.28$, $p<0.01$, $F(\text{time})_{7, 72}=1.44$, NS, and $F(\text{interaction})_{7, 72}=3.83$, NS) or heart rate ($F(\text{treatment})_{1, 72}=7.58$, $p<0.05$, $F(\text{time})_{7, 72}=7.41$, NS, and $F(\text{interaction})_{7, 72}=2.47$, NS) in awake, freely moving animals (Figure 13).

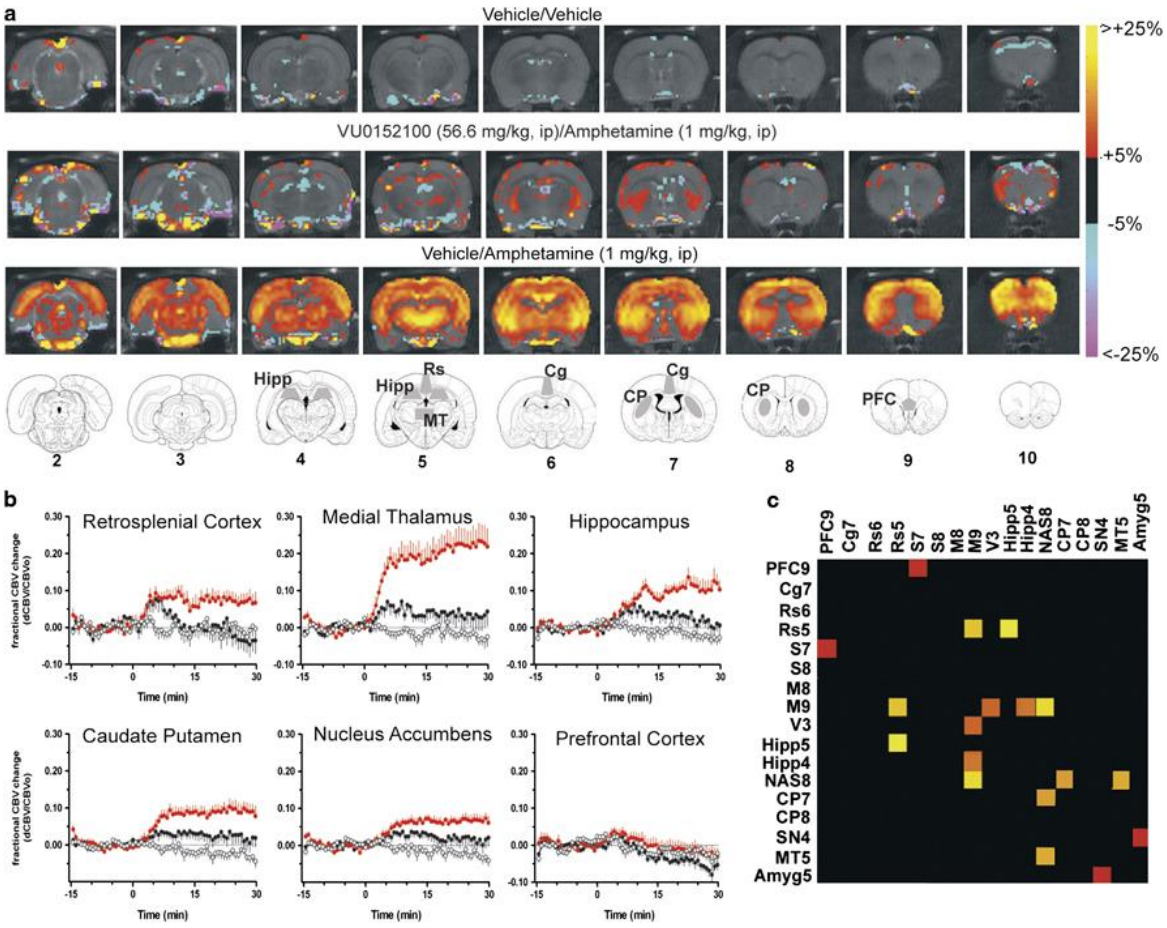


Figure 12. VU0152100 modulates amphetamine-induced cerebral blood volume responses. (a) Amphetamine (1 mg/kg, i.p.) elicited CBV increases, reflecting neural activity, while pre-treatment with VU0152100 (56.6 mg/g, i.p.) suppressed this effect in multiple brain areas. (b) Regional CBV time courses for amphetamine and reversal by VU0152100 are shown for the retrosplenial cortex, medial thalamus, hippocampus, NAS, CP, and PFC; note that amphetamine by itself and in combination with VU0152100 did not alter CBV in the PFC. Data are means±SEM of 10–11 animals per group. Functional connectivity analysis of the phMRI data revealed fewer inter-regional correlations in the VU0152100/amphetamine group and the significant ROI–ROI correlation differences between the vehicle/amphetamine group and the VU0152100/amphetamine group (thresholded at $p<0.05$) are depicted in panel (c). Cells are colored according to the corresponding z-statistic. Abbreviations of regions of interest (number indicates slice): Amyg, amygdala; CP, caudate-putamen; Cg, cingulate; Hipp, hippocampus; MT, medial thalamus; M, motor cortex; NAS, nucleus accumbens; PFC, prefrontal cortex; Pir, piriform cortex; Rs, retrosplenial cortex; S, sensory cortex; SN, substantia nigra; V, visual cortex.

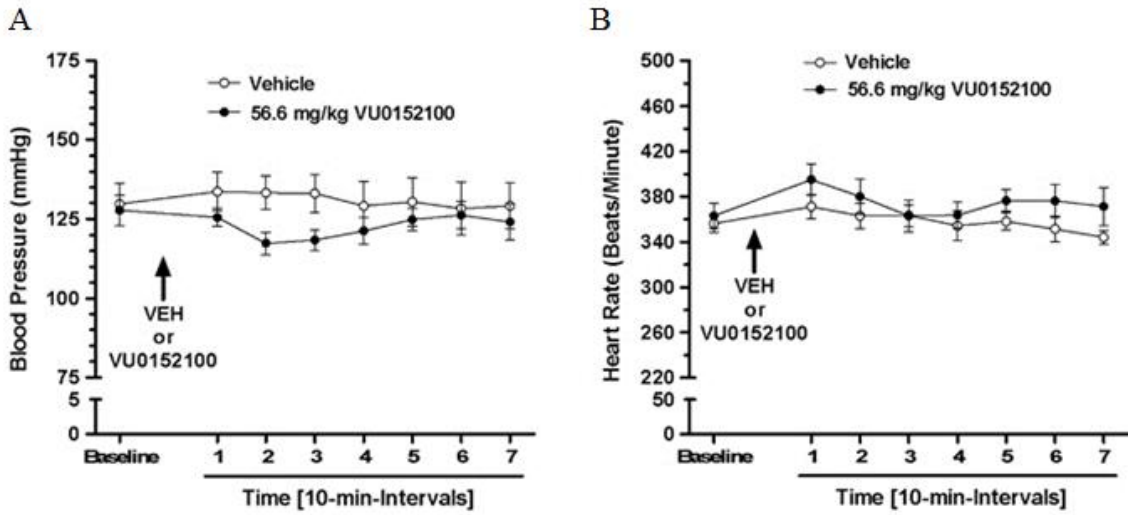


Figure 13. **VU0152100 does not cause cardiovascular side effects**. VU0152100 (56.6 mg/kg, i.p.) does not alter blood pressure and (d) heart rate in awake animals. Although two-way ANOVA revealed a significant treatment effect on blood pressure and heart rate there were we no significant differences between vehicle- and VU0152100-treated rats at any time point (Bonferroni test).

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

The finding that VU0152100 reverses effects of amphetamine in multiple behavioral models and on CBV in multiple brain regions raises the possibility that this M_4 PAM may act in part by reducing effects of amphetamine on extracellular dopamine levels in brain regions associated with antipsychotic efficacy. The effects of VU0152100 in reversing amphetamine-induced increases in extracellular dopamine levels in the NAS and CP were assessed using *in vivo* microdialysis in rats at a dose of 56.6 mg/kg (i.p.), which produced maximum efficacy in blocking amphetamine-induced hyperlocomotion and disruption of PPI. As shown in Figures 14A and B, VU0152100 reversed amphetamine-induced hyperlocomotion ($F(\text{treatment})_{3,\ 312}=30.6$, $p<0.001$, $F(\text{time})_{12,\ 312}=17.5$, $p<0.0001$, and $F(\text{interaction})_{36,\ 312}=34.8$, $p<0.001$; Figure 4b: $F(\text{treatment})_{3,\ 351}=27.1$, $p<0.001$, $F(\text{time})_{12,\ 351}=20.0$, $p<0.0001$, and $F(\text{interaction})_{36,\ 351}=30.7$, $p<0.001$). When administered alone, VU0152100 had no effect on basal dopamine release in either the NAS or CP (Figure 14C and D). However, when given in combination with amphetamine, VU0152100 significantly reduced amphetamine-evoked dopamine release in both the NAS and CP (Figure XC for NAS: $F(\text{treatment})_{3,\ 297}=19.9$, $p<0.001$, $F(\text{time})_{12,\ 297}=23.3$, $p<0.001$, and $F(\text{interaction})_{36,\ 297}=30.2$,$p<0.001$; Figure XD for the CP: ($F(\text{treatment})_{3,\ 351}=18.8$, $p<0.001$, $F(\text{time})_{12,\ 351}=27.3$, $p<0.001$, and $F(\text{interaction})_{36,\ 351}=32.4$, $p<0.001$). In the NAS, VU0152100 alone increased extracellular levels of the dopamine metabolites DOPAC (Figure 15A: $F((\text{treatment})_{3,\ 296}=16.3$, $p<0.001$, $F(\text{time})_{12,\ 296}=3.87$, NS, and $F(\text{interaction})_{36,\ 296}=21.2$, $p<0.001$) and HVA (Figure 15C: $F(\text{treatment})_{3,\ 296}=17.9$, $p<0.001$, $F(\text{time})_{12,\ 296}=2.86$, NS, and $F(\text{interaction})_{36,}$

$_{296}$=17.3,p<0.001). In the CP, amphetamine alone or in combination with VU0152100 reduced DOPAC (Figure 15B: F(treatment)$_{3,\ 334}$=16.4, p<0.001, F(time)$_{12,\ 334}$=11.8, p<0.001, and F(interaction)$_{36,\ 334}$=17.7, p<0.001) and HVA (Figure 15D: F(treatment)$_{3,\ 331}$=13.9, p<0.001, F(time)$_{12,\ 331}$=9.98, p<0.001, and F(interaction)$_{36,\ 331}$=24.0, p<0.001).

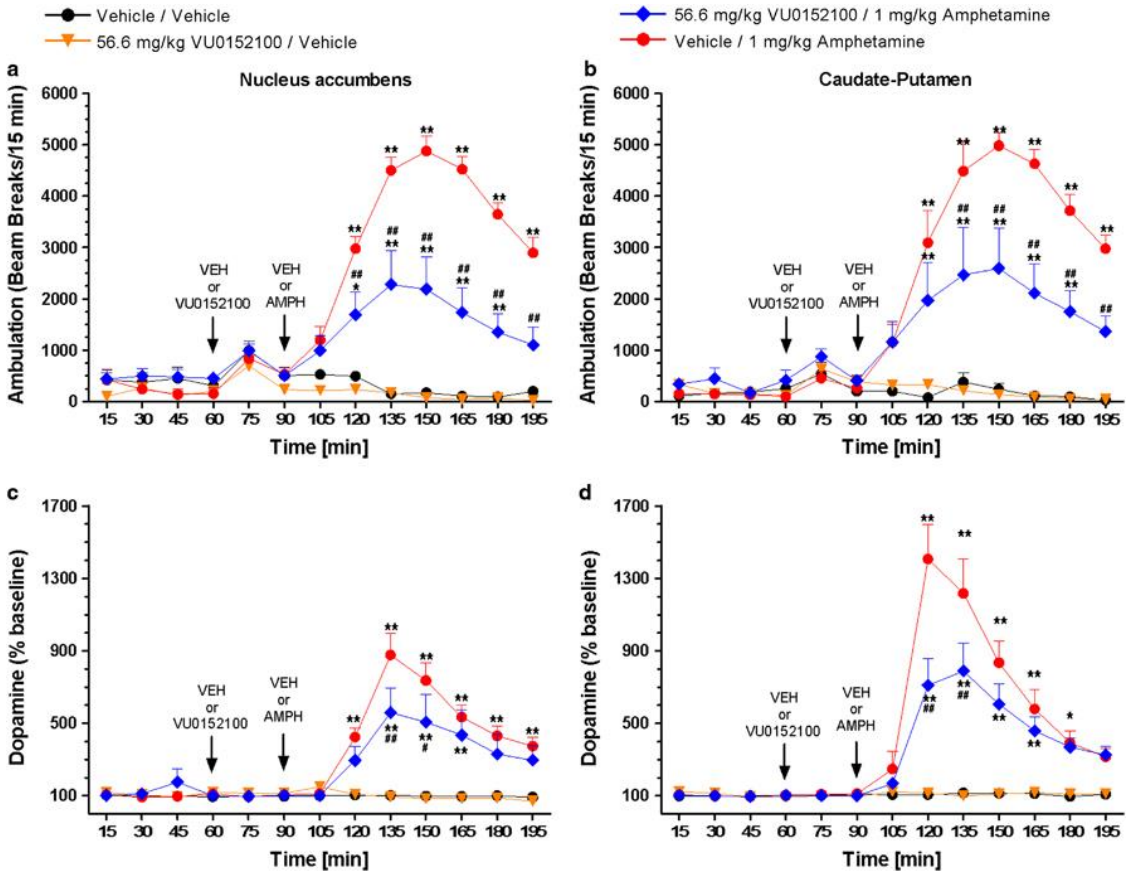


Figure 14. Effects of VU0152100 on locomotor activity and dopamine efflux in the nucleus accumbens and caudate-putamen. Pre-treatment with VU0152100 (56.6 mg/kg, i.p.) reverses amphetamine-induced (1 mg/kg, s.c.) hyperlocomotion (a, b) and reduces the amphetamine-elicited increase in extracellular dopamine levels in the nucleus accumbens and caudate-putamen (c, d). Data are mean±SEM of 6–8 rats per group; *p<0.05, **p<0.01 vs vehicle/vehicle; #p<0.05, ##p<0.01 vs vehicle/amphetamine (Dunnett's test).

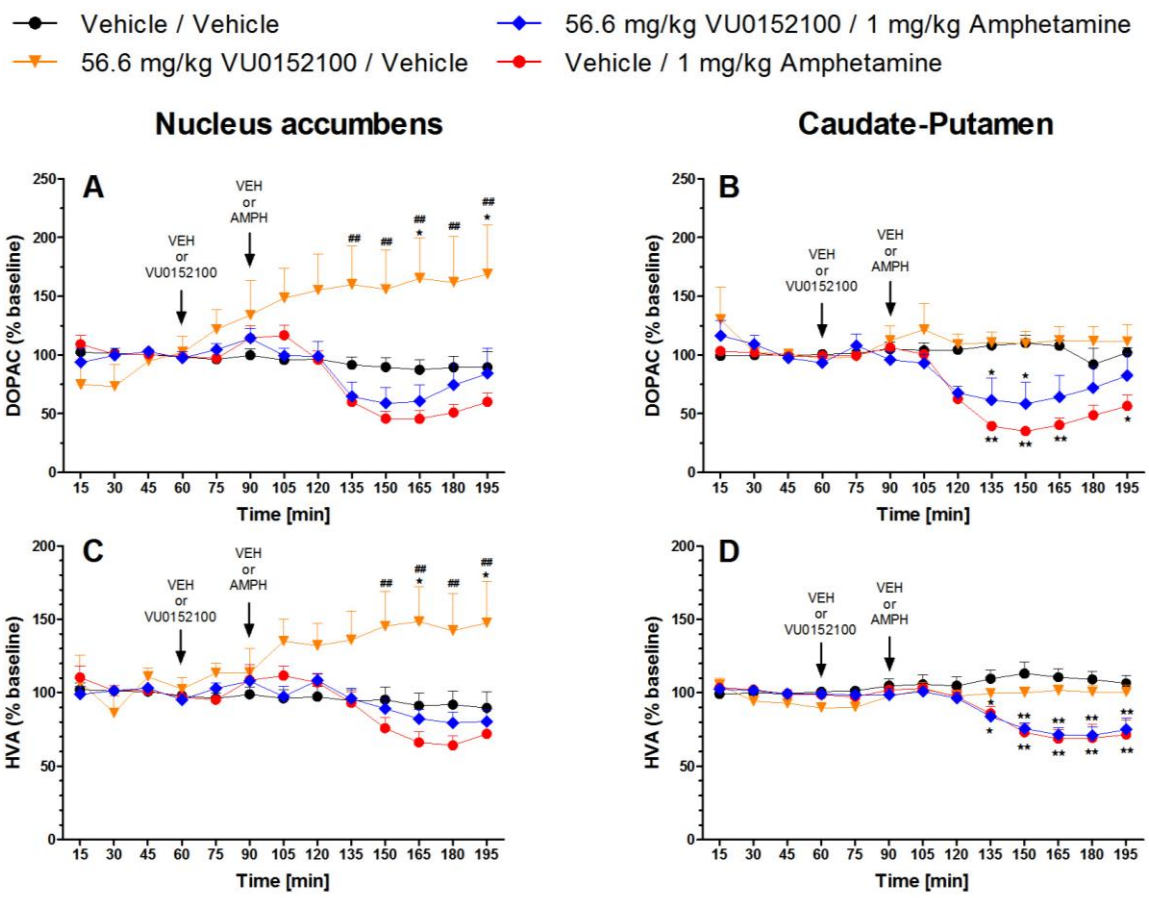


Figure 15. Effects of VU0152100 on extracellular dopamine metabolites. Effects of VU0152100 (56.6 mg/kg, ip) and amphetamine (1 mg/kg, sc), alone or in combination on extracellular levels of the dopamine metabolites DOPAC (A and B) and HVA (C and D) in the nucleus accumbens (A and C) and caudate-putamen (B and D). Data are mean ± S.E.M. of 6 - 8 rats/group; * $p<0.05$, ** $p<0.01$ vs. vehicle/vehicle; # $p< 0.01$ vs. vehicle/amphetamine (Dunnett's test).

Discussion

In recent years, selective activators of mAChRs have emerged as a new approach for the treatment of psychotic symptoms and cognitive disturbances associated with various neuropsychiatric disorders. In these studies, the selective M_4 PAMs VU0152100 and VU0467154 displayed dose-dependent efficacy in behavioral models that predict antipsychotic-like activity. In contrast to orthosteric mAChR agonists, VU0152100 and VU0467154 did not produce adverse effects associated with non-selective activation of peripheral mAChR subtypes (data not shown) or impairments in locomotor activity when administered alone. phMRI studies also indicated that VU0152100 reduces amphetamine-induced activation of the NAS and CP, as well as certain cortical, thalamic, and hippocampal areas. Functional connectivity analyses of the phMRI data revealed that VU0152100 modulated the activity of neural circuits that included the NAS, retrosplenial and motor cortices, hippocampus and thalamus. Finally, the behavioral effects of VU0152100 were accompanied by reversal of amphetamine-induced increases in extracellular DA levels in the NAS and CP, suggesting that the antipsychotic-like activity is mediated, in part, through modulation of mesolimbic and mesostriatal dopamine signaling.

The present findings suggest that VU0152100 and VU0467154 reverse amphetamine-induced behavioral and neurochemical changes directly through potentiation of M_4 mAChRs. Although the observed effects could also be due to a drug–drug interaction between the M_4 PAM and amphetamine, this seems unlikely since drug-naive rats were used in acute dosing paradigms that would not allow sufficient time for induction of metabolizing enzymes responsible for degrading amphetamine to occur

(Fahmi & Ripp, 2010). Moreover, we have previously reported a lack of VU0152100 and VU0467154 interaction with a host of GPCRs, transporters, ion channels, and enzymes found in the periphery and CNS which would be relevant for the observed behavioral responses (Brady et al., 2008; Bubser et al., 2014).

Our finding that VU0152100 attenuates amphetamine-induced increases in extracellular DA in the NAS and CP is consistent with previous studies and suggests that the *in vivo* antipsychotic-like effects of M_4 PAMs are at least partially mediated by reduction of DA release. In particular, DA release in the nucleus accumbens and striatum can be induced by administration of muscarinic agonists, but is absent in M_4 KO mice, suggesting that M_4 negatively regulates dopaminergic tone in these regions (Threlfell et al., 2010). Currently, the mechanism by which M_4 activation regulates mesolimbic and mesostriatal dopaminergic transmission remains unclear. M_4 has been postulated to act as an inhibitory autoreceptor on terminals of cholinergic neurons originating in the pedunculopontine and laterodorsal tegmental nuclei that excite dopaminergic neurons in the ventral tegmental area (Holmstrand & Sesack, 2011; Yeomans, 2012). In this case, M_4 activation in the VTA would result in attenuation of dopaminergic signaling in the mesolimbic pathway. In addition, the observed effects on dopamine signaling could also be the result of M_4 activation on cholinergic interneurons in the NAS, which have been shown to enhance phasic DA release under normal conditions (Cachope et al., 2012). There is also recent evidence suggesting that activation of M_4 receptors results in increased activity in D1-containing striatal projection neurons through a mechanism of action involving enhanced Ca^{2+} currents though Ca_v1-channels (Hernandez-Flores et al., 2015). This, in turn, would result in enhanced GABAergic signaling to the VTA, leading

to reductions in DA release in the striatum. Conflicting reports suggest that M_4 activation in the NAS results in direct inhibitory control of DA D1 receptor signaling, resulting in decreases in inhibitory control of VTA signaling (Onali & Olianas, 2002).

There is emerging evidence implicating interactions between M_4 receptors and the endocannabinoid system, namely through a CB_2-dependent mechanism (Foster et al., 2016). These studies showed that activation of M_4 receptors on striatal spiny projection neurons resulted in dopaminergic regulation resulting in a sustained depression of striatal dopamine release. Furthermore, both the M_4-mediated sustained inhibition of dopamine release and the antipsychotic-like efficacy of M_4 activators were dependent on functional signaling through CB_2 cannabinoid receptors. These findings highlight a novel mechanism by which striatal cholinergic and cannabinoid signaling leads to sustained reductions in dopaminergic transmission, resulting in behavioral effects predictive of antipsychotic-like activity.

Consistent with the behavioral and neurochemistry results, VU0152100 suppressed amphetamine-induced increases in CBV in the NAS and CP as well as the medial thalamus, hippocampus, and retrosplenial cortex. It has been previously reported that changes in blood volume can reflect changes in neural activity, specifically in response to psychoactive compounds (Chen et al., 1997; Hackler et al., 2010; Kim & Ogawa, 2012; Schwarz et al., 2004). The effects of VU0152100 on brain activity in multiple regions suggest that actions in areas outside of the basal ganglia may also contribute to the *in vivo* antipsychotic-like effects of VU0152100. These findings are consistent with previous evidence that cortical projections modulate DA function in the striatum (Desole et al., 1992; Tzavara et al., 2004). We characterized the regional

correlation patterns in response amplitude to VU0152100 using a well-established functional connectivity analyses approach (Schwarz et al., 2007; Schwarz et al., 2007). Pre-treatment with VU0152100 attenuated several strong connectivity patterns of the NAS and retrosplenial cortex with other brain areas after amphetamine challenge. These included correlated activity between NAS and hippocampus, motor cortex, and CP and the retrosplenial cortex with motor cortex and hippocampus. Importantly, the low isoflurane (0.88%) necessary for proper maintenance anesthesia to prevent movement-related artifacts did not result in negative CBV changes to amphetamine. Also, the observed changes in hemodynamics cannot be attributed to changes in blood pressure or heart rate, as VU0152100 administration had no effect on either at any specific time point. Although additional studies are needed to further investigate the underlying mechanisms of the observed functional changes by VU0152100, these data suggest M_4 potentiation can directly and indirectly modulate dopaminergic signaling in multiple brain regions implicated in schizophrenia.

It is important to note that while M_4-mediated decreases in DA release may contribute to the *in vivo* effects of VU0152100, the phMRI findings would suggest other mechanisms are also likely contribute to the antipsychotic-like actions of M_4 PAMs. Previous studies have reported the ability of xanomeline to reverse the behavioral effects of both amphetamine and the direct dopamine receptor agonist apomorphine and that these effects are eliminated in M_4 KO mice (Dencker et al., 2011; Jones et al., 2005; Stanhope et al., 2001). The ability of M_4 activation to inhibit responses to a direct acting dopamine receptor agonist suggests that M_4 activation must also act at a level downstream of DA release to induce its overall *in vivo* effects. With this in mind, it is

worth noting that M_4 receptors are expressed at multiple levels in basal ganglia and forebrain regions, and future studies utilizing VU0152100 or VU0467154 are essential to elucidate other actions of M_4 PAMs that may contribute to the overall *in vivo* effects.

These studies, taken together with a growing body of research discussed previously, suggest that M_4 PAMs reduce dopaminergic signaling and have a range of efficacy in rodent models predictive of antipsychotic-like activity. Importantly, these findings support a role for M_4 in the antipsychotic-like efficacy observed with the M_1/M_4-preferring ligand, xanomeline, and point to modulation of other brain circuits and neurotransmitter systems implicated in the cognitive deficits and negative symptoms observed in schizophrenia. Studies described in Chapters III-V will address these questions in relation to modulation with both M_1 and M_4 PAMs.

Sections of this chapter have been reprinted from the following:

Byun, N.E., Grannan, M., Bubser, M., Barry, R.L., Thompson, A., Rosanelli, J., Gowrishankar, R., Kelm, N.D., Damon, S., Bridges, T.M., Melancon, B.J., Tarr, J.C., Brogan, J.T., Avison, M.J., Deutch, A.Y., Wess, J., Wood, M.R., Lindsley, C.W., Gore, J.C., Conn, P.J., and Jones, C.K. (2014) Antipsychotic drug-like effects of the selective M4 muscarinic acetylcholine receptor positive allosteric modulator VU0152100. *Neuropsychopharmacology*. **39**(7): 1578-93.

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CHAPTER III

SELECTIVE POTENTIATION OF M_4 MUSCARINIC ACETYLCHOLINE RECEPTORS MODULATES ELECTROPHYSIOLOGICAL AND BEHAVIORAL EFFECTS IN PHARMACOLOGIC AND GENETIC MODELS OF NMDAR HYPOFUNCTION

Introduction

Previous studies suggest that acute psychotic episodes in patients diagnosed with schizophrenia are associated with increased activity in several key brain regions, including the PFC (Cleghorn et al., 1989; Ebmeier et al., 1995; Hermle et al., 1992; Kaplan et al., 1993; Vollenweider et al., 1997a; Vollenweider et al., 1997b). In support of this, recent clinical findings have demonstrated that acute treatment with the NMDAR antagonist ketamine in healthy humans can induce a state of mPFC disinhibition, or hyperconnectivity, that is also observed in early course schizophrenic patients (Anticevic et al., 2015). In animal studies, acute pharmacologic challenge with either NMDAR antagonists or hallucinogens induces increases in spontaneous activity at glutamatergic synapses in layer V mPFC in brain slices and *in vivo* (Jackson et al., 2004; Aghajanian & Marek, 1999). One mechanism for this increase in spontaneous activity at mPFC glutamatergic synapses is through direct activation of 5-HT_{2A} receptors located presynaptically on glutamatergic thalamocortical terminals in the mPFC. Upon activation

of these 5-HT_{2A} receptors, there is a dramatic increase the frequency of spontaneous excitatory postsynaptic currents (EPSCs) measured in layer V pyramidal cells of the mPFC (Aghajanian & Marek, 1997; Aghajanian & Marek, 1999; Marek et al., 2001; Marek et al., 2000). Previous studies have demonstrated that atypical antipsychotics, including clozapine and olanzapine, inhibit this effect and reduce spontaneous EPSCs in the mPFC by direct antagonist activity at 5-HT_{2A} receptors on thalamocortical terminals. Based on the postulated role of altered glutamatergic transmission in the pathophysiology of schizophrenia, this has been suggested to be important for the unique clinical profile of atypical antipsychotic agents (Deutch et al., 1991). More recently, novel ligands with antipsychotic-like activity, such as metabotropic glutamate receptor subtypes 2 and 3 ($mGlu_2$ and $mGlu_3$) agonists and $mGlu_2$ potentiators, have been shown to dramatically reduce increases in spontaneous EPSCs induced by 5-HT_{2A} receptor activation in mPFC pyramidal cells. Additional studies showed this inhibition of glutamate signaling was dependent on activation of presynaptic $mGlu_2$ receptors expressed on thalamocortical nerve terminals (Jackson et al., 2004; Aghajanian & Marek, 1999). Importantly, administration of these ligands also resulted in a reduction of hyperactive glutamatergic signaling *in vivo*, suggesting modulation of this circuit is critical for mPFC function (Lorrain et al., 2003; Lorrain et al., 2003; Moghaddam & Adams, 1998).

In the following studies, we performed experiments to elucidate the role of M_4 mAChRs in cortical circuits thought to be disrupted in schizophrenia. To do this we utilized a range of electrophysiological techniques, both *ex vivo* and *in vivo*, along with behavioral assays known to be dependent on cortical glutamatergic signaling.

Additionally, the effects of VU0467154 were assessed in a genetic model of NMDAR hypofunction (introduced in Chapter I and discussed in great detail in Chapter V) to confirm a role for M_4 modulation in glutamatergic signaling in a model of constitutive, global NMDAR deficient mice.

Methods

Animals

All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All *in vivo* electrophysiology studies were carried out using adult male Sprague–Dawley rats (Harlan, Indianapolis, IN). *Ex vivo* electrophysiology studies used age-matched male WT and M_4 KO mice (P25-30) 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.

Compounds

5-amino-3,4-dimethyl-*N*-(4-((trifluoromethyl)sulfonyl)benzyl)thieno[2,3-*c*]pyridazine-6-carboxamide (VU0467154) was synthesized in house as previously described (Bubser et al., 2014). For *in vivo* studies, VU0467154 was dissolved in a 10%

tween 80 vehicle and injected intraperitoneal (i.p.). *Ex vivo* electrophysiology studies used VU0467154 dissolved in a dimethyl sulfoxide (DMSO) stock, then diluted to 3 μM in artificial cerebrospinal fluid (aCSF).

Whole-Cell Patch-Clamp Recordings

Whole-cell patch-clamp recordings were performed using 300 μm coronal slices prepared from P25-P30 day old WT and M_4 KO mice with a K-gluconate-based intracellular solution as described previously (Shirey J. Neuro). Spontaneous EPSCs were recorded from pyramidal cells in layer V prelimbic mPFC while clamped at -70 mV. The interevent intervals of sEPSCs from 2 min episodes during baseline and drug application were used to generate cumulative probability plots. The interevent intervals from each experiment were then expressed as frequency and the mean values from the 2 min episodes were grouped and compared. Inward current data analysis was performed using Clampfit and Minianalysis software.

Stereotaxic Injections

Viral-mediated gene transfer of channelrhodopson-2 (ChR2) in the midline nuclei of the thalamus (mThal) was accomplished in male C57bl/6j mice (4-5 weeks of age). Anesthesia was obtained via dexmetomedine-ketamine (0.5-80 mg/kg, i.p.), after which mThal was targeted at ML: ± 0.3, AP: -1.2, DV: -3.0, relative to bregma with a stereotaxic instrument and software (Leica Angle Two). Bilateral injections of AAV5-CaMKII-ChR2-EYFP (250-400 nL; UNC Viral Vector Core) were delivered at 100 nL/min. Atipamazole (1.0 mg/kg, s.c.) and ketoprofen (10 mg/kg, s.c.) were administered

to reverse anesthesia and relieve pain. Mice were sacrificed for *ex vivo* electrophysiology 3-4 weeks post-surgery.

Whole-cell Optogenetic Electrophysiology

Mice were anaesthetized with isoflurane and decapitated. The brains were rapidly removed and placed in cold sucrose-based slicing solution (in mM): 183 sucrose, 20 NaCl, 0.5 KCl, 2.0 CaCl2, 1.0 MgCl2, 1.2 NaH2PO4, 10 glucose, and 26 NaHCO3. Hemisected coronal slices (250 μM) were prepared using a Leica VT 1200S vibratome and immediately transferred for 10 minutes to a heated (37 ± 1 °C) bath containing N-methyl-D-glucamine (NMDG)-based recovery solution (in mM): 93 NMDG, 20 HEPES, 2.5 KCl, 0.5 CaCl2, 10 MgCl2, 1.2 NaH2PO4, 25 glucose, 30 NaHCO3, 5 Na-ascorbate, and 3 Na-pyruvate. Slices then recovered for at least 60 min at room temperature (23 ± 1 °C) in a holding chamber containing artificial cerebrospinal fluid (aCSF) (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl2, 1.3 MgCl2, 1 NaH2PO4, 11 glucose, and 26 NaHCO3. Once placed in the recording chamber, slices were continuously perfused with aCSF at a rate of 2 ml/min at 30 ± 2°C. Picrotoxin (50 μM) was added to the recording aCSF to block GABAARs. All solutions were continuously oxygenated with 95% O2 / 5% CO2.

Whole-cell voltage-clamp recordings were obtained using IR-DIC video microscopy. Recordings were made with 3.0–4.0 MΩ glass electrodes pulled on a P-1000 Flaming/Brown puller (Sutter Instruments) filled with (in mM): 120 CsMeSO4, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 MgATP, 0.3 Na3GTP, 0.1 spermine, and 5 QX-314. For optogenetic experiments, ChR2-driven optical excitatory postsynaptic currents (op-EPSCs) were elicited by 1-2 ms illumination with 470 nm light. Recordings

were acquired using a Multiclamp 700B (Molecular Devices) amplifier, filtered at 2 kHz and digitized at 10 kHz. The monosynaptic component of the op-EPSC was defined to be the peak current obtained in the 4-8 ms window following stimulation. The light intensity was adjusted to obtain op-EPSCs with a magnitude of 150–400 pA. Cells were voltage clamped at -70 mV and op-EPSCs were evoked at 0.1 Hz. Data acquisition and analysis were performed using pClamp 10.4 software (Axon Instruments). Membrane properties were monitored continuously throughout the duration of experiments, and changes in series resistance were less than 20 % across each experiment. op-EPSCs were binned by minute and subsequently normalized to the average value of the 5-minute baseline. The coefficient of variation (CV) of 30 responses was determined by dividing the standard deviation the mean.

RNAScope

Animals received 125 nl deposits of cholera toxin B-Alexa 555 into the medial prefrontal cortex (N=4); injections involved the prelimbic cortex (area 32) and the dorsally contiguous anterior cingulate cortex (area 24b). 7-10 days later animals were overdosed with isoflurane, the brains removed and blocked, and tissue stored at -80°C until assayed. Coronal sections were cut at 14um through the frontal cortex and thalamus and collected on Superfrost slides. Chrm4 transcripts were labelled using the Advanced Cell Diagnostics (Newark, CA) RNAscope fluorescent assay kit (ACD Manual Cat. No. 320293) with the following modifications. The sections were fixed in 4% paraformaldehyde in phosphate buffer at 4°C for 15 minutes, after which there were dehydrated for 1 min each in 50%, 50%, 70%, 100% and 100% ethanol. Sections were

treated with 2 drops of RNAscope Pretreat 4 for 15 minutes at room temperature and rinsed in PBS. For the hybridization step, 50 µL Chrm4 probe set/section was added, and 1 drop/section of the AMP used.

(±) DOI Head Twitch

On test day, the animals were taken to the testing room and allowed to habituate for up to 60 minutes. Animals received an intraperitoneal (IP) injection of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, Sigma) directly before testing. Animals were dosed with the M_4 PAM VU0467154 or vehicle 60 minutes prior to DOI dose. The mGlu2 PAM BINA was used as a positive control and was dosed 30 minutes prior to DOI. After the injection, each animal was placed in a large glass beaker for 30 minutes. A head-twitch response is characterized by a distinct left-to-right or right-to-left rapid head movement following DOI treatment. Head twitches were recorded by video continuously over the course of 30 minutes in a quiet room. Scoring was completed by 2 independent, blinded scorers and the data was averaged.

Locomotor Activity Studies in Mice

Spontaneous locomotor activity was assessed in 8-10 week old wild-type and NR1 KD mice. Animals were placed in an open field system (OFA-510, MedAssociates, St. Albans, VT) with three 16 × 16 arrays of infrared photobeams as described previously (Byun et al., 2014). Mice were allowed to habituate in their home cage for one hour prior to the start of the experiment. Following the habituation period, mice were injected with either vehicle (10% tween 80, i.p.) or a dose of VU0467154 (3 mg/kg or 10 mg/kg, i.p.)

and placed directly into the open field chambers. Spontaneous locomotor activity was recorded for 120 minutes. The time course of drug-induced changes in spontaneous locomotor activity is expressed as distance traveled (cm) per 5 minute bin for the 90 minute session. Total activity data represent the sum of all 5 minute bins for the recording session. Data are represented as mean ± SEM and were analyzed using two-way ANOVA with Bonferroni's post hoc test.

***In vivo* electrophysiology**

Male Sprague-Dawley rats (300-400g) were anaesthetized with 1-2% isoflurane and secured using a stereotaxic device. Upon completion of craniotomy and dural opening, five quartz-platinum/tungsten electrodes were independently driven into the mPFC (-3.0 mm AP; +1.0 mm ML; -3.0 mm DV). Once stable baseline recording was achieved, rats were dosed with either VU0467154 (10 mg/kg, IP) or vehicle (10% tween 80), followed 30 minutes later by dosing with either MK-801 (0.1 mg/kg, SC) or vehicle (sterile water). Continuous recording occurred for 60 minutes following the final injection. Offline sorting was performed to determine cell type. Only cells that produced a minimum of 100 spikes in the baseline sample with less than 1% refractory period violations (refractory period <1.1 ms) were used for subsequent analysis. Pyramidal cells were defined as neurons with firing rate < 5 Hz with sporadic firing patterning. The firing rate of each neuron was normalized to the baseline frequency and neurons were grouped based on the drug treatment the aniamal received. Changes in firing rate (frequency) were determined using Neuroexplorer V4.091 (Nex Technologies, Madison, AL).

Spatial Alternation

Briefly, mice were initially trained to respond in either left or right nosepoke, with the correct nosepoke alternating after each training session for two to three sessions in each nosepoke. The response contingencies were then changed such that the mice were required to respond alternately on the left and right nosepoke within each training session to obtain 33% ensure liquid reinforcer. The beginning of each trial was signaled by the illumination of the stimulus light in the nosepoke hole. Correct responses produced a liquid reinforcer and initiated a 10-second retention interval, illumination of the house light, and extinction of the stimulus light. During the retention interval responses had no scheduled consequences. Incorrect responses were followed by a 0.2-second feedback tone. After an incorrect trial, the correct nosepoke remained on the same side; i.e., a correction procedure was used. The retention interval was held constant for 15 training sessions, at which time responding asymptoted at approximately 85% correct. The duration of the retention interval was then varied among 2, 4, 8, 16, and 32 seconds in a randomized order during each session. All response contingencies remained the same. Behavior was allowed to stabilize over the next 15 sessions. Sessions were conducted 5 days per week and each training session ended after 60 minutes or 100 reinforcers, whichever occurred first.

***In Vivo* Electrophysiology**

The method was a modified version of that described in (Walker et al., 2008). Mice anesthetized with isoflurane were secured on a stereotaxic apparatus and a craniotomy was performed at AP(+1.7 mm) ML(+0.5 mm). The electrode bundle was

composed of eight 25 μm Formvar-insulated stainless-steel wires for recording single units and two 50 μm uninsulated stainless-steel ground wires, one of which served as a depth reference electrode. The bundle length was 2.5 mm, allowing for recording of prelimbic mPFC pyramidal cells when the array is fixed to the skull with dental acrylic. Mice are allowed to recover 5-7 days before entering the recording arena. All recordings took place between 12pm and 4pm. A wire harness attached to a headstage containing unity gain amplifiers was secured to the electrode array and mice were allowed to explore the chamber for fifteen minutes prior to the initiation of baseline recording. Animals were placed in a chamber equipped with a Faraday cage and attached to an electric commutator which allowed for unrestricted movement during the *in vivo* recording.

Neuronal discharges were acquired by the Multichannel Acquisition Processor (MAP) system following preamplification (Plexon Inc, Dallas, TX). To detect spiking activity, signals were bandpass filtered (154 Hz to 8.8 kHz) and digitized at a rate of 40 kHz. After establishing a voltage threshold ≥2.5 times background noise, waveforms were continuously collected and principal component analysis was used to discriminate putative pyramidal neurons from fast-spiking interneurons. All recording sessions lasted for 30 minutes. Baseline firing rate was assessed during the final 5 minutes of the baseline collection period (30 minutes into recording), and the effects of vehicle (10% tween 80, i.p.) or VU0467154 (10 mg/kg , i.p.) were assessed during the final 5 minutes of the drug treatment period (60 minutes following dose). Data are expressed as mean frequency during each period or as a percent of baseline and significance is determined using a paired t-test.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism 5 (GraphPad, San Diego, CA). The effects of pharmacological application in whole cell electrophysiology experiments were compared using unpaired t-test. DOI-induced head twitch and *in vivo* electrophysiology studies were compared using two-way analysis of variance (ANOVA) with Bonferroni post hoc tests unless otherwise mentioned.

Results

VU0467154 attenuates 5-HT-induced increases in EPSCs in layer V mPFC pyramidal cells in WT, but not M_4 KO tissue

In animal studies, acute pharmacologic challenge with either NMDAR antagonists or hallucinogens induce increases in spontaneous activity at glutamatergic synapses in layer V mPFC in brain slices and *in vivo* (Aghajanian & Marek, 1999; Jackson et al., 2004). One mechanism for this increase in spontaneous activity at mPFC glutamatergic synapses is through direct activation of 5-HT2A receptors located presynaptically on glutamatergic thalamocortical terminals in the mPFC. Previous reports provide evidence for modulating thalamocortical circuitry via activation or potentiation of presynaptic receptors expressed on this circuit (Gewirtz & Marek, 2000). To model disruptions in this circuit *ex vivo*, 5-HT application was utilized to elicit changes in glutamatergic signaling. Bath application of 5-HT (30 µM) for 10 minutes resulted in a robust increase (153.0 ± 9.638 percent of baseline; Figure 16D and E) in sEPSCs in WT tissue, resulting in a leftward shift in the interevent interval cumulative probability plot consistent with previously reported results (Benneyworth, 2007). Co-application of VU0467154 (3 µM)

significantly reduced the response to 5-HT (97.19 ± 17.37 percent of baseline; Figure 16F and G) resulting in a rightward shift in the cumulative probability plot towards baseline values. In contrast, 5-HT application alone or in combination with VU0467154 had no effect on amplitude of sEPSCs as depicted in the representative cumulative probability plot and group means ($p > 0.05$; Figure 16F and G).

Identical studies were performed using M_4 KO mouse tissue in order to determine if the effects of VU0467154 observed in WT tissue were M_4-mediated. Bath application of 5-HT (30 μM) still elicited a leftward shift in the interevent interval cumulative probability plot and a significant increase in sEPSC frequency compared to baseline (161.3 ± 5.663 percent of baseline; Figure 17D and E); however, co-application with VU0467154 had no effect on the 5-HT-induced response. Similar to WT tissue, neither 5-HT alone or in combination with VU0467154 had a significant effect on sEPSC amplitude (Figure 17F and G). Taken together, these data indicate VU0467154 modulates glutamatergic signaling in layer V mPFC in an M_4-dependent manner.

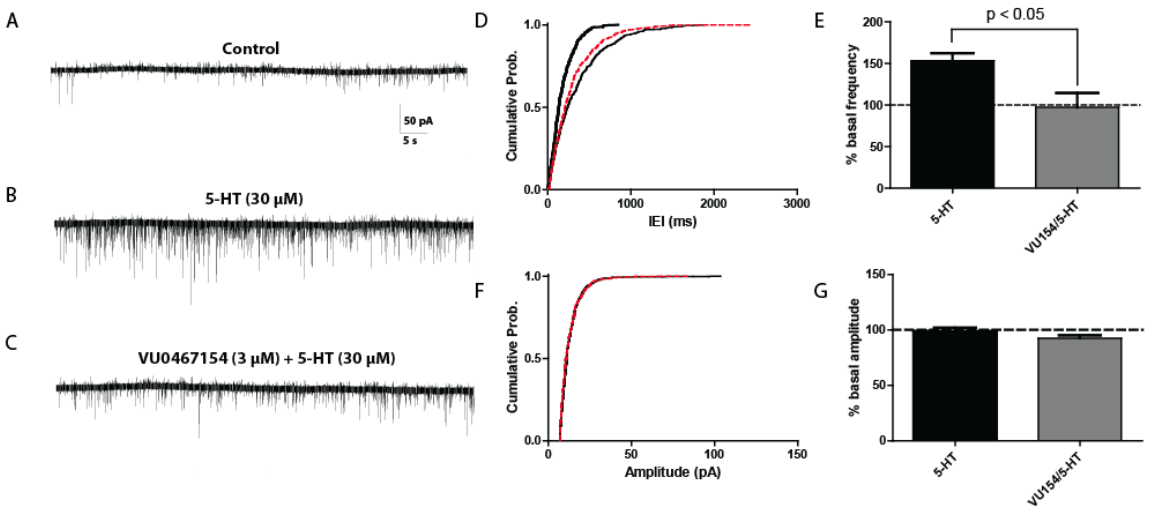


Figure 16. 5-HT-induced increases in sEPSCs can be attenuated with co-application of VU0467154 in WT tissue. (A-C) Representative EPSC traces from WT slices in layer V mPFC pyramidal cells. (D) Representative cell showing 5-HT bath application (30 µM) causes a leftward shift in the interevent interval cumulative probability plot (bold line). Co-application with VU0467154 (3 µM) attenuates this response resulting in a rightward shift (red dotted line). (E) 5-HT bath application induces robust increase in EPSCs, but co-application with VU0467154 reverses this response (n=5-7). (F) Representative cell showing no change in amplitude cumulative probability plot following 5-HT or 5-HT + VU0467154 co-application. (G) 5-HT or 5-HT + VU0467154 bath application did not result in a significant change in amplitude of EPSCs compared to baseline. (paired two-tail t test; p value <0.05).

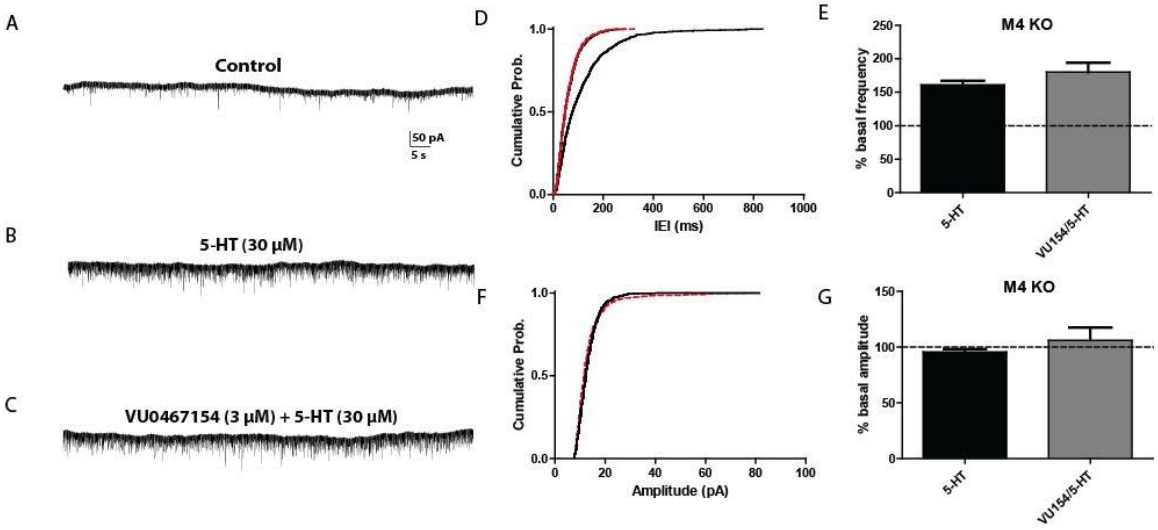


Figure 17. 5-HT-induced increases in sEPSCs are not attenuated with co-application of VU0467154 in M_4 KO tissue. (A-C) Representative EPSC traces from M_4 KO slices in layer V mPFC pyramidal cells. (D) Representative cell showing 5-HT (30 µM) bath application induces a significant leftward shift in the interevent interval cumulative probability plot in M_4 KO tissue (bold line). VU0467154 (3 µM) co-application had no effect on IEI (red dotted line). (E) 5-HT application resulted in a significant increase in sEPSCs, an effect that could not be attenuated with VU0467154 co-application in M_4 KO tissue (n=5-7). (F,G) 5-HT and 5-HT + VU0467154 co-application had no effect on amplitude of sEPSCs as observed in the representative cumulative probability plot and averaged group mean. (paired two-tail t test; p value <0.05).

VU0467154 attenuates thalamocortical synaptic transmission

Viral-mediated gene transfer of ChR2-EYFP in the midline thalamic nuclei led to robust expression of ChR2-EYFP in the PFC and the medial striatum (Figure 18A). Optically-evoked excitatory postsynaptic currents (op-EPSCs) were elicited from layer V PFC pyramidal neurons. Application of NBQX blocked op-EPSCs (data not shown), confirming AMPAR-mediated glutamatergic transmission. After obtaining a stable baseline, VU0467154 (3 μM) was bath applied and a significant reduction in the amplitude of the op-EPSC 10-15 minutes after drug application (Figure 18B-C; 82.7 ± 2.5 % baseline, $p < 0.05$, one-sample t-test) was observed. Of note, no electrical stimulation was applied during these experiments, consistent with the effect of VU0467154 on spontaneous neurotransmission. In the PFC, M_4 receptors may be expressed on pyramidal neurons and/or as presynaptic heteroreceptors on thalamic inputs. To begin to address the synaptic locus of VU0467154 action, we analyzed the coefficient of variation (CV), which is inversely correlated with presynaptic release probability. VU0467154 application increased the CV of the evoked responses (0.181 ± 0.021 vs. 0.142 ± 0.024 at baseline, $p < 0.05$, paired t-test, Figure 18E), consistent with the interpretation that potentiation of M_4 receptors inhibits thalamocortical neurotransmission through a presynaptic site of action.

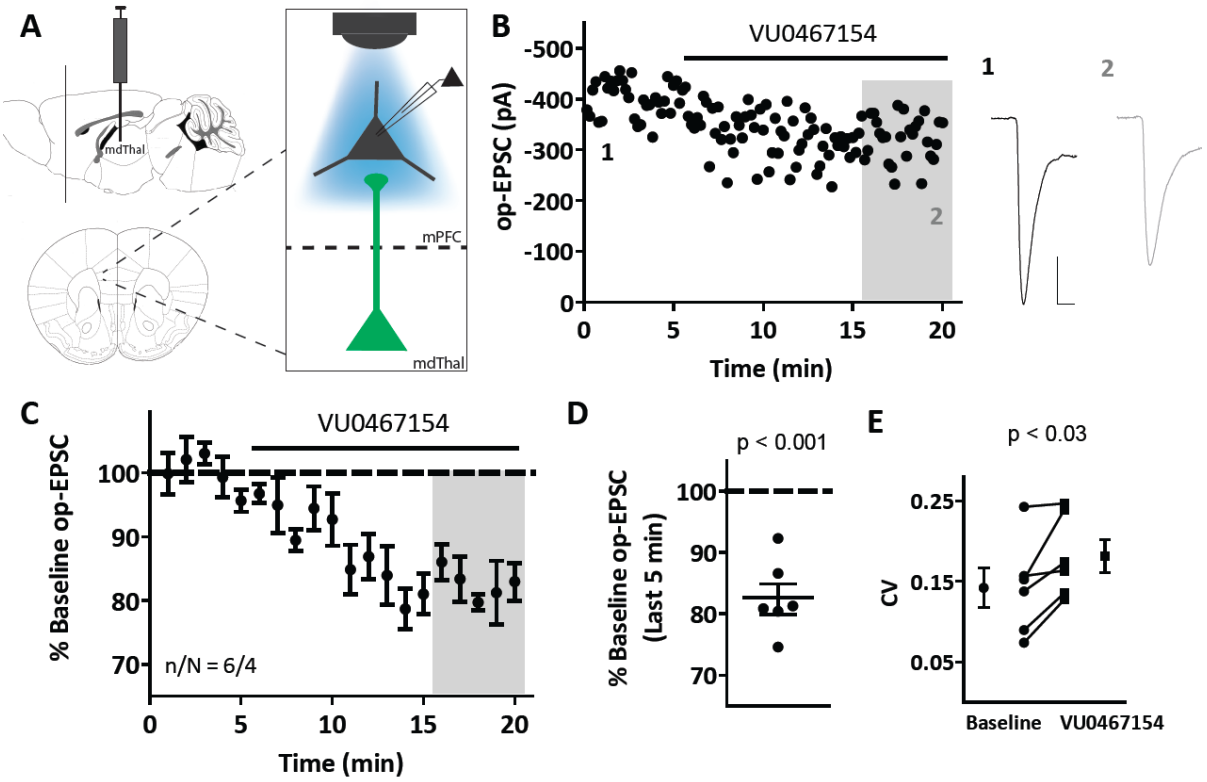


Figure 18. VU0467154 attenuates thalamocortical synaptic transmission. (A) Images of ChR2-EYFP expression and experimental schematic. ChR2-EYFP was exogenously expressed in the midline thalamus and terminal expression was observed in the PFC 3-4 weeks later. Light stimulation elicited op-EPSCs from neurons in the PFC. (B) Left, representative experiment displaying timecourse of op-EPSC amplitude following bath application of VU0467154 (3 µM,). Right, representative op-EPSC traces during baseline (black) and drug application period (gray). Scale bars denote 5 ms and 100 pA. (C) Summarized data displaying timecourse of VU154-mediated op-EPSC inhibition (n = 6 cells, N = 4 mice). (D) Scatter plot showing average op-EPSC amplitude in final 5 minutes of VU0467154 application normalized to baseline (gray area panel B,C). (E) VU0467154 application increased the coefficient of variation (CV) of the op-EPSC.

VU0467154 administration reverses NMDAR antagonist-induced increases in pyramidal cell firing rate in layer V mPFC

Consistent with previous reports, systemic dosing with the NMDAR antagonist MK-801 (0.1 mg/kg, i.p., vehicle saline) induced a robust increase in pyramidal cell firing rate in layer V mPFC neurons that persisted for 30 minutes following dosing (Figure 19). Pretreatment with VU0467154 (10 mg/kg, i.p, vehicle 10% tween 80, 30 minute pretreatment time) resulted in a complete blockade of this response. Dosing with VU0467154 (10 mg/kg, i.p, vehicle 10% tween 80, 30 minute pretreatment time) alone did not affect firing rate.

VU0467154 Attenuates Excessive Pyramidal Cell Firing in the Prefrontal Cortex of NR1 KD Mice.

In vivo electrophysiology recordings from the prelimbic cortex of awake, behaving mice revealed a significant increase in firing rate of pyramidal neurons in NR1 KD mice (1.70 Hz ± 0.14) compared to wild-type littermate controls (1.040 Hz ± 0.06)(t_{129} = 4.49, $p < 0.0001$; see Figure 29B). Mice received either vehicle or 10 mg/kg VU0467154 following 30 minutes of baseline recording (Figure 20A). Pyramidal cell firing rate was reduced in the NR1 KD animals following VU0467154 administration (61.08% ± 4.59, t_{63} = 3.70, $p < 0.005$; Figure 20D), with no observed effect of vehicle dosing (101.7% ± 10.6; Figure 20D). Vehicle administration did not affect firing rate in wild-type animals, though VU0467154 treatment did produce a significant reduction (107.8% ± 4.75 and 85.5% ± 6.55, respectively, t_{63}= 2.64, $p < 0.05$; Figure 20G).

Comparison of raw firing rates using a paired *t*-test revealed a strong post-dose decrease in NR1 KD mice following VU0467154 administration (t_{34}= 7.68, $p < 0.0001$; Figure 20B) with a much smaller decrease in wild-type animals (t_{33}= 2.70, $p < 0.05$; Figure 20E).

(±) DOI induced head-twitch response can be reversed by VU0467154

(±) DOI-induced head-twitch response onset was observed approximately 5 minutes after (±) DOI administration. Pretreatment with VU0467154 dose-dependently reversed head-twitch response (ANOVA, Dunnett's test, ** $p<0.01$, *** $p < 0.001$ *vs.* vehicle + DOI, $F_{6,38}$=10.07, Figure 21). Pretreatment with 56.6 mg/kg of the mGlu2 PAM BINA resulted in a significant reduction in head-twitch response that is consistent with previously reported literature (Benneyworth, 2007) (ANOVA, Dunnett's test,*** $p < 0.001$ *vs.* vehicle + DOI, $F_{6,38}$=10.07). (±) DOI administration in M_4 KO mice resulted in comparable head-twitch responses to those observed in WT littermate controls, but the effect of the top dose of 10 mg/kg VU0467154 was absent (unpaired t-test, $p = 0.5734$, $t_{0.5788, 12}$).

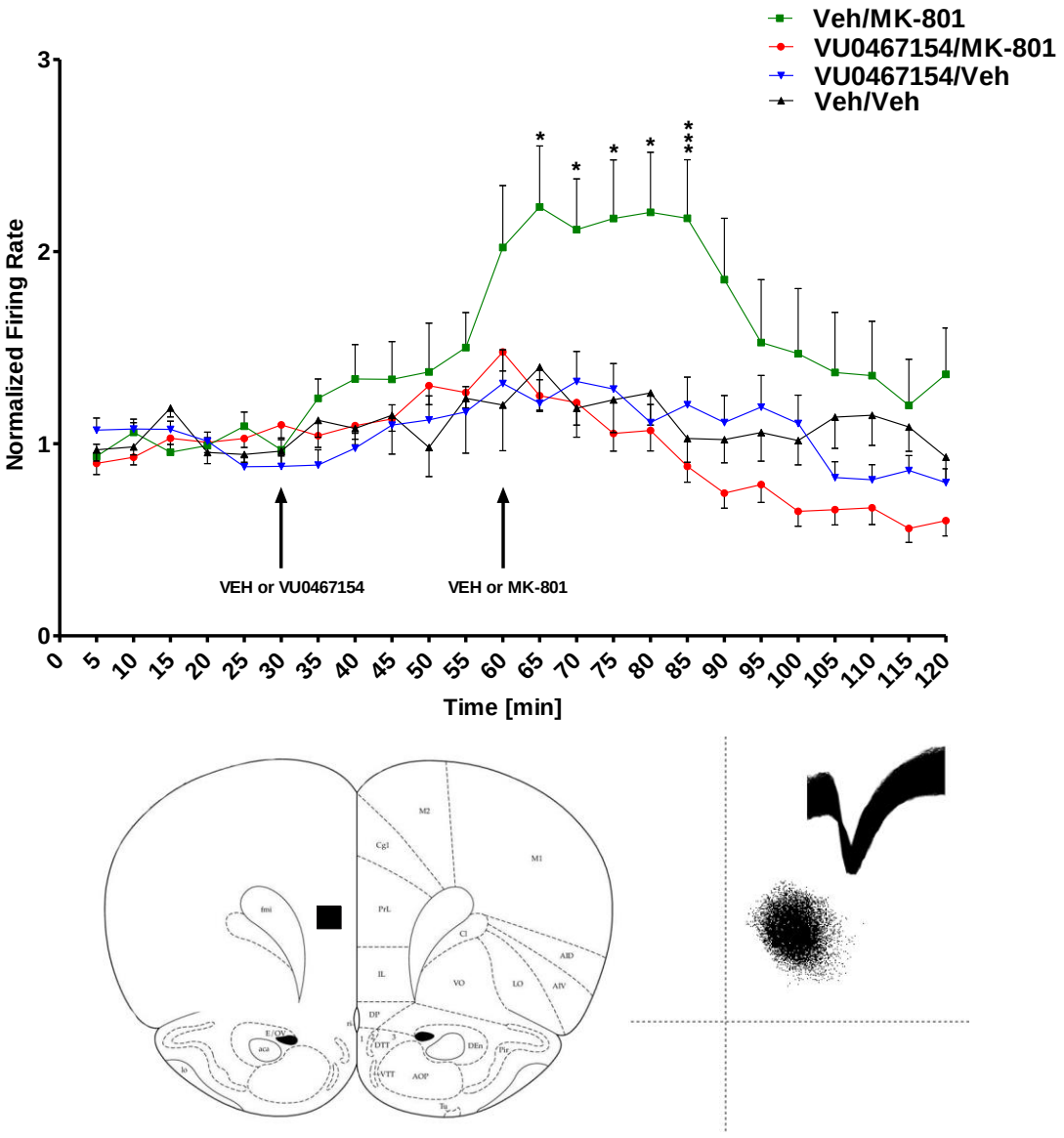


Figure 19. NMDAR antagonist systemic administration results in enhanced PFC pyramidal neuron firing rate. MK-801 administration results in increased pyramidal cell firing rate in layer V mPFC in rat (squares). Pretreatment with VU0467154 significantly reverses MK-801-induced increases in firing rate. VU0467154 administration alone had no effect on firing rate. Data are mean±SEM of 6-8 rats per treatment group, 40-60 cells. (vehicle 10% Tween 80, VU0467154 10 mg/kg, IP, 60 min pretreatment; 0.1 mg/kg MK-801, SC, 30 min pretreatment, ANOVA, Bonferroni's test * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$ *vs.* vehicle +vehicle). Below (left), schematic showing location of electrode placement location in layer V PrL cortex (black square). Below (right), representative neuron graphed in principle component analysis space along with representative waveforms.

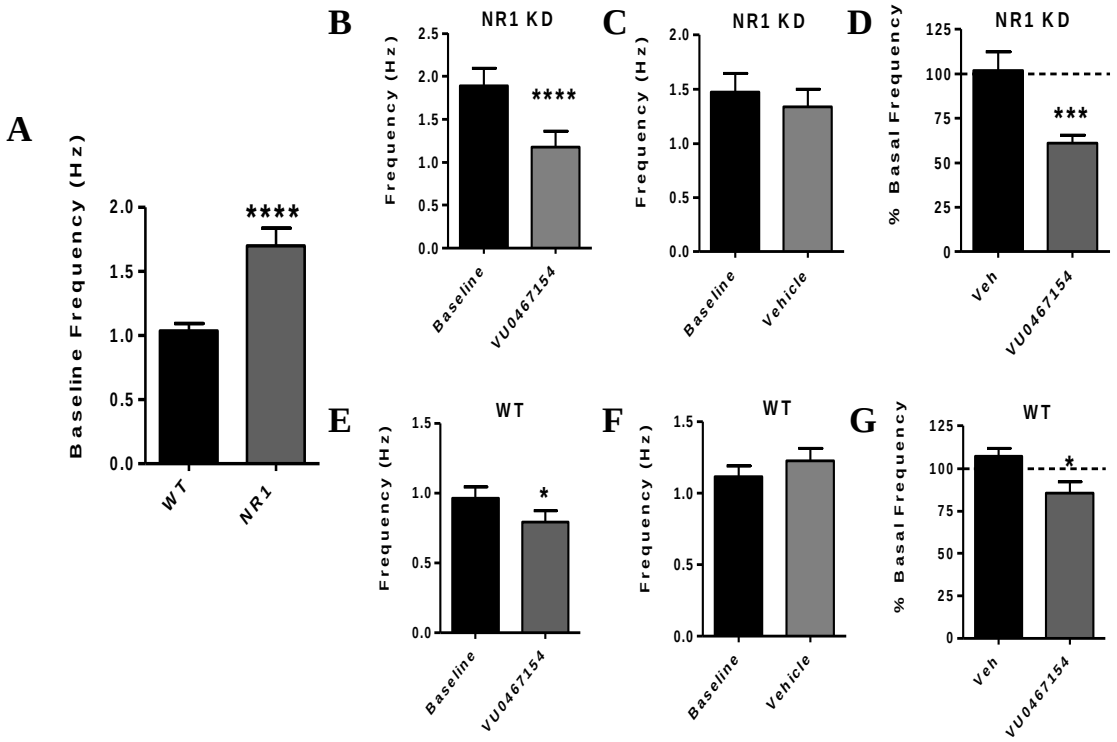


Figure 20. VU0467154 reduces pyramidal cell firing rate in awake, freely moving NR1 KD mice. (A) NR1 KD mice exhibit significantly increased firing rate compared to WT littermate controls. (B-D) VU0467154 significantly reduced pyramidal cell firing rate in NR1 KD mice, but vehicle treatment had no effect. (E-F) VU0467154 also reduced pyramidal cell firing rate WT littermate controls. Data in panels D and G represent changes in frequency compared to the baseline recording period prior to drug administration. (A, D, and G) t-test, *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$. (b, C, E, and F) Paired t-test, *$p < 0.05$, ****$p < 0.001$. A: n=65-66 cells, B-G: n=30-35 cells.

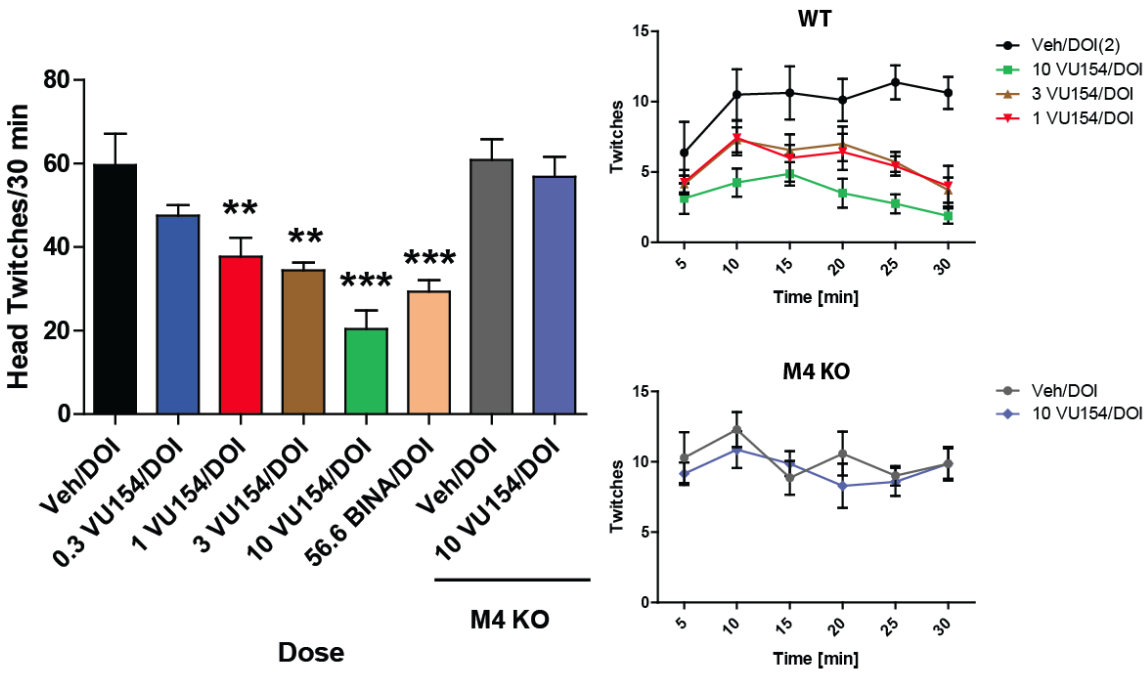


Figure 21. (±) DOI induced head-twitch response can be reversed by VU0467154. VU0467154 dose-dependently reverses (±) DOI-induced head-twitch response in WT mice, but not M_4 KO mice. (Right) Time-course of drug effect in WT and M_4 KO mice. (vehicle 10% Tween 80, VU0467154 & BINA IP, 60 min pretreatment VU0467154, 30 min pretreatment BINA; 2.0 mg/kg (±) DOI, IP, ANOVA, Dunnett's test * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$ *vs.* vehicle +DOI; M_4 KO Veh/DOI vs. 10 VU154/DOI n.s, Unpaired t-test).

VU0467154 administration reverses NMDAR antagonist-induced deficits in an operant-based spatial alternation task

WT mice were trained in an operant-based spatial alternation task. Upon acquisition of stable baseline, characterized as 2 consecutive training days above 80%, mice were dosed with the following paradigm: vehicle/vehicle (10% tween 80, i.p., 60 min prior to test; sterile water, i.p., 30 min prior to test), vehicle/MK-801 (10% tween 80, i.p., 60 min prior to test; MK-801, 0.3 mg/kg, i.p., 30 min prior to test) or VU0467154/MK-801 (VU0467154, 10 mg/kg, i.p., 60 min prior to test; , MK-801, 0.3 mg/kg, i.p., 30 min prior to test). MK-801 treatment resulted in a significant disruption in performance compared to vehicle administration (Figure 22) (42.6 ± 3.5% vs. 85.2 ± 1.5%, respectively; $F_{(2,29)} = 43.76$, $p < 0.0001$). Pretreatment with VU047154 significantly attenuated the response to MK-801 alone, resulting in performance above chance responding (63.1 ± 3.8% vs. 42.6 ± 3.5%, respectively).

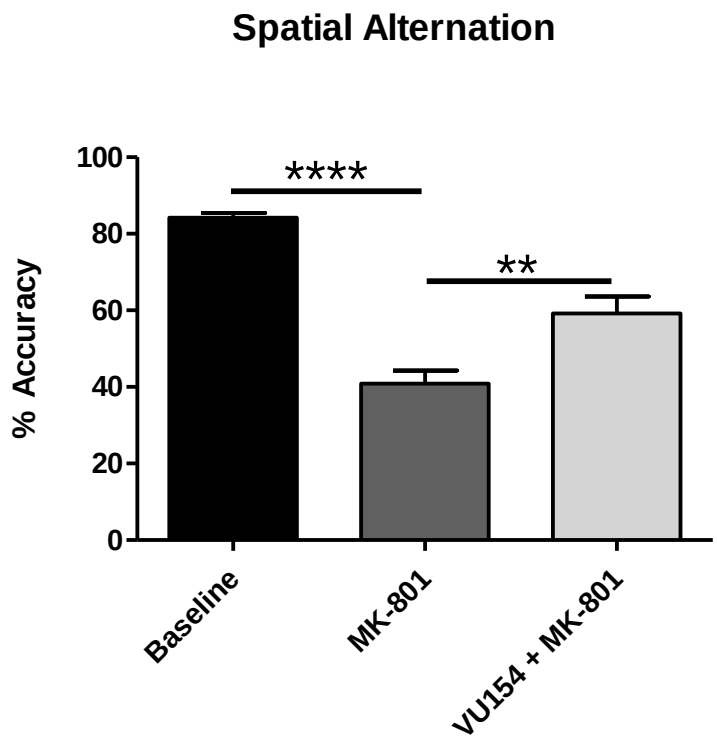


Figure 22. M_4 PAM administration reverses NMDAR-induced deficits in spatial alternation performance. MK-801 (0.3 mg/kg, i.p.) administration significantly reduced accuracy in an operant-based spatial alternation task. Pretreatment with VU0467154 (10 mg/kg, i.p.) resulted in a significant attenuation of this deficit. **p < 0.01, ****p < 0.0001, one-way ANOVA, Bonferroni's post hoc test.

Chrm4 mRNA is found in mediodorsal thalamic projection neurons to the mPFC

Most but not all cells of the mediodorsal nucleus of the thalamus (MD) expressed Chrm4; the cholinergic receptor mRNA was also observed in the thalamic paraventricular and intermediodorsal nuclei, and in the paracentral nucleus posteriorly (Figure 23). Almost all cells retrogradely labeled from the PFC expressed Chrm4. Expression levels of the cholinergic receptor transcript were subjectively moderate, being roughly comparable to the CA1 of the hippocampus and the retrosplenial cortex overlying the MD.

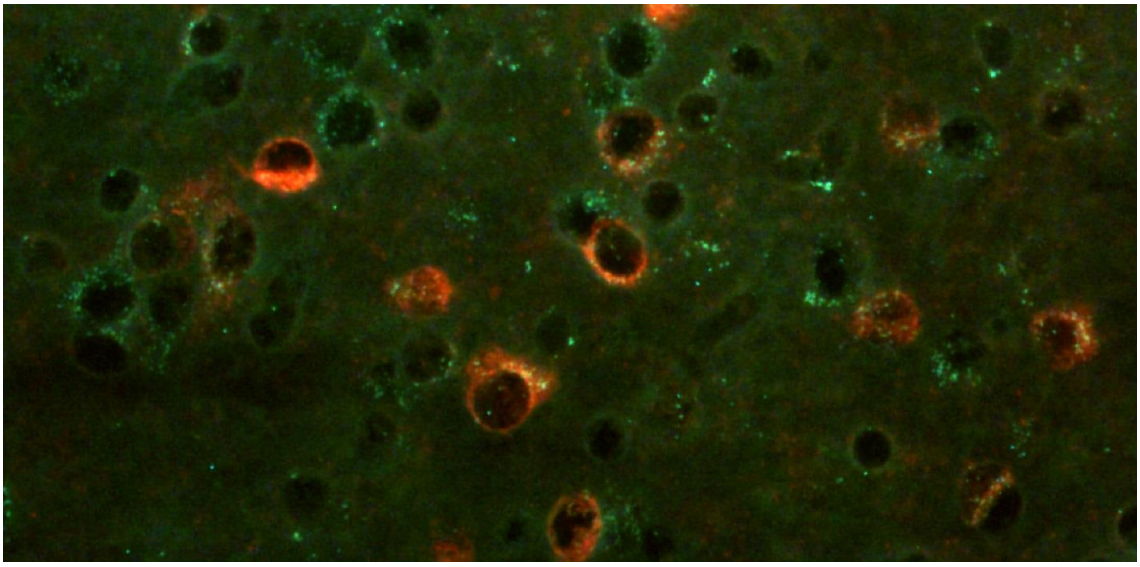


Figure 23. **Chrm4 mRNA is found in mediodorsal thalamic projection neurons to the mPFC.** Both MD neurons that project to the PFC (retrogradely labeled with CTB, in red) and MD neurons that do not innervate the PFC (open nuclei) express *Chrm4* (green puncta). Few if any of the thalamocortical neurons do not express the receptor transcript.

Discussion

In Figure 16, we measured spontaneous excitatory postsynaptic currents (sEPSCs) in layer V mPFC pyramidal cells using whole-cell voltage clamp electrophysiology. Bath application of serotonin (5-HT) in this paradigm results in increased sEPSCs via enhanced presynaptic glutamate release from thalamocortical terminals (Aghajanian & Marek, 1999). We hypothesized that M_4 modulation on these terminals could potentially regulate glutamate release in the cortex, which was observed when VU0467154 was co-applied with 5-HT. This response was absent in tissue from M_4 knockout animals (Figure 17). These exciting data are consistent with previous reports by our group and others demonstrating that the selective $mGlu_2$ PAM BINA and $mGlu_{2/3}$ agonists also blocked increases in the frequency of sEPSCs in layer V pyramidal cells of the PFC induced by activation of $5\text{-}HT_{2A}$ receptors on these presynaptic glutamatergic thalamocortical terminals in the PFC (Benneyworth et al., 2007).

To confirm a role for M_4 on thalamocortical terminals we recorded optically-evoked excitatory postsynaptic currents (op-EPSCs) from layer V PFC pyramidal neurons following viral-mediated gene transfer of ChR2-EYFP in the midline thalamic nuclei before and after VU0467154 application (Figure 18), where M_4 potentiation lead to a significant decrease in peak amplitude. To begin to address the synaptic locus of VU0467154 action, we analyzed the coefficient of variation (CV), which is inversely correlated with presynaptic release probability. VU0467154 application increased the CV of the evoked responses consistent with the interpretation that potentiation of M_4 receptors inhibits thalamocortical neurotransmission through a presynaptic site of action.

Based on our findings in whole cell electrophysiology we hypothesized MK-801 administration *in vivo* would result in dramatic changes in mPFC electrophysiological properties. To test this hypothesis we dosed anesthetized rats with MK-801 and observed changes in firing rate of putative pyramidal neurons in layer V mPFC (Figure 19). Pre-treatment with VU0467154 was able to completely block this NMDAR antagonist-induced increase in relative firing rate. These data are consistent with our hypothesis that administration results in a disinhibition of the thalmocortical circuit, and likely others, but that M_4 potentiation is sufficient to dampen the resulting changes in network excitability *in vivo*.

Here we report deficits in spontaneous pyramidal cell activity in the layer V PFC of awake, free-moving NR1 KD mice in comparison with their wild-type littermate controls. These findings revealed substantial increases in cortical pyramidal cell activity that are consistent with previous studies performed in rodents after acute administration of MK-801 or PCP, and which are known to be associated with deficits in cognitive performance and information-processing (Jackson et al., 2004). At present, the underlying mechanism(s) for this disinhibition of PFC activity in the NR1 KD mice remains unclear. However, based on NMDAR antagonist studies, one possible explanation may involve increased PFC activation resulting from disinhibition of thalamocortical circuitry, leading to excessive glutamate release and cortical excitability (Celada et al., 2013). If disruptions in normal signaling in this circuit are contributing to the observed changes in cortical electrophysiology, it is likely that VU0467154 administration is attenuating this response based on the studies outlined above. However, there are additional mechanisms by which and M_4 PAM could be eliciting these effects.

Previous reports indicate increases in activity in hippocampal-PFC inputs following NMDAR antagonist treatment (Jodo, 2013; Jodo et al., 2005; Kamiyama et al., 2011; Thomases et al., 2014). This, taken with reports that M_4 PAMs can modulate hippocampal synaptic transmission (Shirey et al., 2008), suggests modulation of this circuit could also contribute to the observed efficacy in NR1 KD mice. Future studies will be conducted to directly investigate the potential for M_4 PAMs to modulate thalamocortical and hippocampal-cortical in NR1 KD mice.

Previous reports indicate excessive glutamatergic signaling in the prefrontal cortex in response to hallucinogen administration manifests as a stereotyped head twitch response in mice that is distinct from other stereotypical movements, such as rearing, ear scratching, and sniffing (Schreiber et al., 1995). This is thought to be the result of activation of 5-HT_{2A} receptors expressed presynaptically on thalamocortical afferents, demonstrated by loss of effect in 5-HT_{2A} knockout mice, in the presence of 5-HT_{2A} antagonists, and in thalamocortical lesion models (Gewirtz & Marek, 2000; Willins & Meltzer, 1997; Klodzinska et al., 2002). In light of the *in vivo* and *ex vivo* electrophysiology data, experiments were designed to examine the effects of an M_4 PAM in behavioral models of hyperglutamatergic signaling. As Figure 20 demonstrates, the 5-$HT_{2A/2C}$ agonist (±) DOI elicits a robust increase in head twitch response following systemic administration. This response can be significantly attenuated with VU0467154 pretreatment, suggesting M_4 modulation is sufficient to affect glutamate signaling. Importantly, this response is consistent with previous reports that the $mGlu_2$ PAM BINA can attenuate the head twitch response through a presynaptic mechanism (Benneyworth et al., 2007). The effects of VU0467154 are absent in M_4 knockout mice confirming the

observed behavioral responses are M_4-dependent. To further assess the ability of an M_4 PAM to influence behavioral changes in response to hyperglutamatergic signaling in the PFC, an operant-based spatial alternation task was performed in WT mice. Spatial learning tasks have been shown to heavily rely on normal signaling in the mPFC, and pharmacologic manipulations or lesions that disrupt this activity result in deficits in performance (Floresco et al., 1997; Kolb, 1984; Sargolini et al., 1999; Seamans et al., 1995; Sutherland et al., 1982).

Taken together, our data offer new evidence to support a broader role for M_4 modulation in the neural circuits mediating the affective and cognitive functions that are disrupted in neuropsychiatric disorders such as schizophrenia.

CHAPTER IV

ENHANCED ACQUISITION OF A VISUAL TOUCHSCREEN PAIRWISE DISCRIMINATION TASK FOLLOWING REPEATED DOSING WITH THE M_4 PAM VU0467154

Introduction

Current antipsychotic medications (APDs) for schizophrenia primarily treat positive symptoms (hallucinations, delusions) yet are largely ineffective in treating the negative (anhedonia, social withdrawal, apathy) and cognitive symptoms (attention, memory, executive function) (Nuechterlein et al., 2004; APA, 2000; Barch & Ceaser, 2012). Overall functional outcome in patients with schizophrenia has been positively linked to cognitive function (Bobes et al., 2007; Green et al., 2004; Green, 1996), suggesting that novel treatments to improve cognition may provide greater efficacy compared to currently available APDs. Accumulating evidence suggests that modulation of the muscarinic cholinergic system represents a potential target for developing novel APDs for the treatment of multiple symptom associated with schizophrenia (Jones et al., 2012). Of the five different subtypes of muscarinic acetylcholine receptors (mAChRs), termed M_1-M_5 (Bonner et al., 1987; 1988), M_1 and M_4 mAChRs subtypes demonstrate substantial promise for antipsychotic-like potential (Jones et al., 2012). In clinical studies, the M_1/M_4-preferring mAChR agonist xanomeline reduced the psychotic symptoms and trended to improve cognition in patients with schizophrenia (Shekhar et al., 2008).

However, xanomeline, similar to other mAChR agonists, failed in clinical development due to dose-limiting adverse side effects attributed to nonspecific activation of peripheral mAChRs (McArthur et al., 2010).

Targeting allosteric binding sites that are topographically distinct and less highly conserved than the classic orthosteric binding site represent one pharmacological approach for developing highly selective compounds for individual mAChR subtypes (Conn et al., 2009). Allosteric modulation does not activate a receptor directly, but can potentiate the response of a specific receptor subtype to ACh, thereby enhancing activity-dependent signaling (Conn et al., 2009). We have recently identified and characterized the antipsychotic-like potential of several positive allosteric modulators (PAMs) targeting the M_4 mAChR subtype (Bubser et al., 2014; Byun et al., 2014; Brady et al., 2008). Selective M_4 PAMs produce an APD-like profile in preclinical models of positive and cognitive symptoms as well as alter sleep architecture in a manner that may be beneficial in patients with schizophrenia (Brady et al., 2008; Bubser et al., 2014; Byun et al., 2014; Gould et al., 2016). Specifically, the M_4 PAM VU0467154 reverses amphetamine and MK-801-induced hyperlocomotion, and MK-801-induced excessive increases in high frequency cortical gamma power (Bubser et al., 2014; Gould et al., 2016). MK-801, an antagonist of the *N*-methyl-D-aspartate subtype of the glutamate receptor (NMDAR), models NMDAR hypofunction, which is hypothesized to underlie many of the symptoms in schizophrenia (Coyle et al., 2012; Blot et al., 2013; Anticevic et al., 2015). Although the M_1 mAChR subtype has long been thought to be important for cognition, increasing evidence supports the role of M_4 mAChRs for learning and memory. Importantly, we recently demonstrated that the M_4 PAM VU0467154 blocks

MK-801 induced memory impairments and produces cognitive enhancing effects acutely when administered alone in conditioned freezing assays (Bubser et al., 2014). However, the effects of M_4 PAMs on more complex assessments of learning and memory, and importantly, effects of repeated dosing remain unknown.

In the present studies, we examined the effects of repeated dosing with the M_4 PAM VU0467154 on learning a touchscreen-based visual pairwise discrimination task. We examined both rate of learning by comparing daily percent accuracy across 10 consecutive testing days and acquisition, as defined by the number of days to achieve 80% accuracy. When dosed prior to each discrimination session, VU0467154 dose-dependently enhanced rate of learning and acquisition. VU0467154 administered for 10 consecutive days attenuated MK-801-induced hyperlocomotion to a similar degree as a single administration, and plasma and brain concentrations following repeated dosing were similar to a single dose administration. Lastly, the cholinergic system has been implicated in multiple stages of learning and memory including acquisition or initial encoding as well as stabilization or consolidation of memories over longer periods of time, often associated with sleep-dependent mechanisms (Gais & Born, 2004; Hasselmo & Sarter, 2011). We examined M_4 mAChR-mediated effects on acquisition versus consolidation by comparing administration of VU0467154 before or immediately after cognition sessions. Dosing immediately after cognition sessions produced qualitatively similar cognitive enhancing effects as dosing prior to each session, suggesting effects on cognition may in part be through consolidation of memory. Together these data further support a role for M_4 mAChR in learning and memory and in the potential treatment of multiple symptom clusters associated with schizophrenia.

Methods

Subjects. All behavioral studies were conducted with adult male wild-type mice (n=132) and M_4 KO mice (n=17) with the same genetic background (C57BL/6NTac). Mice were group-housed 2-5 mice per cage in a temperature and humidity controlled environment under a 12/12 h light-dark cycle with water available *ad libitum*. 8-12 week old mice were used for all studies and gradually food restricted and maintained at ~85% free-feeding weight prior to initiating all cognition studies. Cognition sessions occurred during the first 6 hr when the lights were on. All experiments have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health were approved by the Vanderbilt University Animal Care and Use Committee.

Touchscreen training. Mice were trained in operant chambers (Lafayette Instruments, Lafayette, IN) to respond to stimuli presented on a computer screen by breaking an infrared beam in close proximity to the stimuli (e.g. a nosepoke) according to convention (for detailed methods see Horner et al., 2013; Bubser et al., 2014; Gould et al., 2015). Throughout training and testing, a mask was placed over the touchscreen such that responses could only be made in one of two (2x2 inch) windows on the screen. In brief, mice were trained over the course of 7-10 days to initiate each trial by emitting a response via a nose-poke in a receptable located on the wall opposite the touchscreen, turn, and to nose-poke on a stimulus appearing in one of the response windows, and then collect a liquid reward (33% diluted Ensure; 30 µl delivered via a peristaltic pump) from the same receptacle located on the opposite wall from the touchscreen. Trial availability

was signaled by illumination of a light within the receptacle, with a 5 sec inter-trial interval. Incorrect responses (nose-poke on a window without a stimulus) were signaled by extinguishing the houselight for 5 sec. Once mice reliably tracked and responded on the stimuli (>80% accuracy), they were assigned to treatment groups counterbalanced across the following variables based on the last day of training before initiation of the pairwise discrimination task: weight, accuracy, session length, correct response latency (average duration of time from trial initiation to a registered nosepoke on the stimulus) and reinforcer retrieval latency (average duration of time to make a head entry into the reward receptacle following a correct response)

Pairwise Discrimination Task. To examine the role of M_4 mAChR function on learning and memory processing we examined acquisition of a pairwise discrimination task. Following training mice were exposed to 10 consecutive daily cognition sessions in which two stimuli were pseudo-randomly presented on the left and right windows of the screen. Responding on one stimulus (S+, "fan") resulted in delivery of a liquid reward followed by a 5 sec inter-trial interval (ITI); responding on the incorrect stimulus (S-, "marbles") terminated the trial, extinguished the house light, and initiated the 5 sec ITI before the house light illuminated again to signal the next trial. "Correction trials" were not implemented following incorrect responses. Total sessions lasted 60 trials or a maximum of 60 min. For all cognition studies, effects of treatment (dose or dose group) were determined by a Two-Way ANOVA followed by a Dunnett's or Tukey's test comparing treatment to vehicle groups (Exp 1, 4) or comparing all groups to each other (Exp 3), respectively.

Experiment 1. Assessing role of M_4 mAChRs on rate of learning and acquisition of a pairwise discrimination task. Following training wild-type mice were administered vehicle (10% Tween 80 in sterile H_2O) or the M_4 PAM VU0467154 (0.3, 1, 3 or 10 mg/kg, synthesized within the Vanderbilt Center for Neuroscience Drug Discovery; (Bubser et al., 2014)) intraperitoneally (i.p.) 60 min prior to the start of each discrimination session for 10 consecutive days starting on day 1 of the pairwise discrimination task. Doses were based on previous studies showing cognitive enhancing effects or reversal of pharmacological-induced cognitive disruptions (Bubser et al., 2014).

To assess effects of repeated dosing of VU0467154 on plasma and brain concentrations, a subset of mice were administered vehicle or VU0467154 (1, 3, 10 mg/kg) for 4 additional days after the last cognition session (14 days of dosing). 2.5 hr following administration on day 14 mice were lightly anesthetized with isoflurane, decapitated and trunk blood was collected and stored on ice in EDTA-coated blood collection tubes until centrifuged (10 min 3,000 RPM, 4 °C). Brains were extracted and flash frozen on dry ice. Plasma was collected and plasma and whole brain were stored at -80°C until analysis. Total plasma and brain concentrations of VU0467154 were determined using LC-MS/MS methods as previously described (Bubser et al., 2014). Collection time point and procedures were identical to prior studies to compare present data with previously published data following acute administration (Bubser et al., 2014). Calculated unbound plasma and brain concentrations were determined based on plasma (0.022) and brain free fractions (0.014) (Bubser et al., 2014). Data are presented as mean +/- standard deviation (n=4-5/dose).

Experiment 2. Assessing effects of acute (1 day) versus repeated (10 days) dosing of VU0467154 on MK-801-induced hyperlocomotion. To determine if the pharmacodynamic effects of VU0467154 were altered by repeated dosing, we examined the ability of VU0467154 to attenuate MK-801-induced hyperlocomotion, a frequently used assay with predictive validity of antipsychotic-like activity. (Bubser et al., 2014; Anticevic et al., 2015; Coyle et al., 2012). Separate groups of mice were administered vehicle (n=23) or 10 mg/kg VU0467154 (n=12) for 9 consecutive days. On the following day (Day 10), half of the mice in the vehicle group were administered vehicle (n=11) and the other half was administered 10 mg/kg VU0467154 (acute group). The group that received 10 mg/kg VU0467154 for days also was administered 10 mg/kg VU0467154 on Day 10 (repeated dosing group). Effects of acute (1 day) versus repeated administration (10 days) of VU0467154 on MK-801-induced hyperactivity were examined using the following paradigm: Open field activity was tested using an open field system (OFA-510, MedAssociates, St. Albans, VT) with three 16 × 16 arrays of infrared photobeams as described previously (Bubser et al., 2014; Byun et al., 2014). Wild-type mice were habituated for 90 min in the open field before being injected with vehicle or 10 mg/kg VU0467154; 30 min later 0.3 mg/kg MK-801 were administered, and locomotor activity was recorded for an additional 120 min. Doses of VU0467154 and MK-801 were chosen based on previously determined full dose-response functions (Bubser et al., 2014). The time course of drug-induced changes in ambulation was expressed as total distance travelled (cm)/5 min over the entire 240-min session as well as total distance travelled in the 120 min following administration of MK-801. Total activity data (means±S.E.M.)

were analyzed by a one-way ANOVA and *post hoc* comparisons were made by Bonferroni's test.

Experiment 3. Confirming selectivity of VU0467154 for M_4 mAChRs by examining rate of learning and acquisition of a pairwise discrimination task in wild-type and M_4 KO mice. To confirm that the cognitive enhancing effects of VU0467154 were mediated by M_4 mAChRs separate groups M_4 KO mice were administered vehicle or 3 mg/kg VU0467154 (dose producing largest cognitive enhancing effects in wild-type mice) prior to each of 10 consecutive cognition test days.

Experiment 4. Assessing role of M_4 mAChRs on acquisition versus consolidation of memory. To determine whether cognitive enhancing effects of VU0467154 were mediated by enhanced acquisition versus consolidation of memory, wild-type mice were trained as above and then separate groups were administered vehicle or 1 mg/kg VU0467154 60 minutes prior to the start or immediately upon completion of each discrimination session for 10 consecutive days starting on day 1 of the pairwise discrimination task. This dose was chosen as the lowest dose to improve cognition in Experiment 1 and based on a 4.7 elimination half-life following intraperitoneal dosing in mice, would be eliminated within 24 hr. Half of the vehicle-treated mice were dosed before, and the other after each session; percent accuracy between these vehicle-treated groups were not different and averaged together.

Results

Experiment 1. Assessment of the effects of acute (1 day) versus repeated (10 days) dosing of VU0467154 on reversal of MK-801-induced hyperlocomotion. As shown in Figure 24, pretreatment with a dose of 10 mg/kg VU0467154 administered once ($p<0.05$) and after 10 days of repeated once-daily dosing significantly ($p<0.01$) attenuated MK-801-induced hyperlocomotion when summed across the 120 minute period following MK-801 administration ($F_{2,32}=6.91$, $p<0.01$); activity was not different between the two different VU0467154-treatment groups.

Experiment 2. Assessment of the effects of repeated (10 days) dosing of VU0467154 on rate of learning and acquisition of a pairwise discrimination task in wildtype mice. As shown in Figure 25A, on day 1 percent accuracy for all groups was near 50% (chance). For the overall data analysis, 4 of the total 59 mice tested were excluded; 2 mice that completed <20 trials per day and 2 mice that did not improve above 55% accuracy across the 10 days. Over the 10-day dosing period, there was a significant increase in percent accuracy ($F_{9,450}=71.48$; $p<0.0001$), and a significant interaction between day and group ($F_{36,450}=1.92$; $p<0.01$), but not a significant effect of dose ($F_{4,50}=2.52$; $p=0.053$). Dunnett's post-hoc analysis revealed that mice treated with 1 and 3 mg/kg VU0467154 demonstrated a significant improvement in rate of learning, as shown by significantly higher percent accuracy on day 5 (1 mg/kg) and days 5 and 6 (3 mg/kg), compared to the vehicle-treated wildtype mice (all $p<0.05$). Daily IP administration of 0.3 and 10 mg/kg VU467154 did not improve rate of learning in wildtype mice compared to respective vehicle-treated mice, demonstrating a classic inverted-U-shaped dose-effect curve (see below). To examine acquisition between treatment groups, the percent of each group that

acquired per test day (>80% accuracy) was plotted as a survival curve. As shown in Figure 25B, 50% and 80% of the mice treated with 1 and 3 mg/kg VU0467154 acquired this task in 5 and 6 days respectively, compared to 7 and 8 days for vehicle-treated mice.

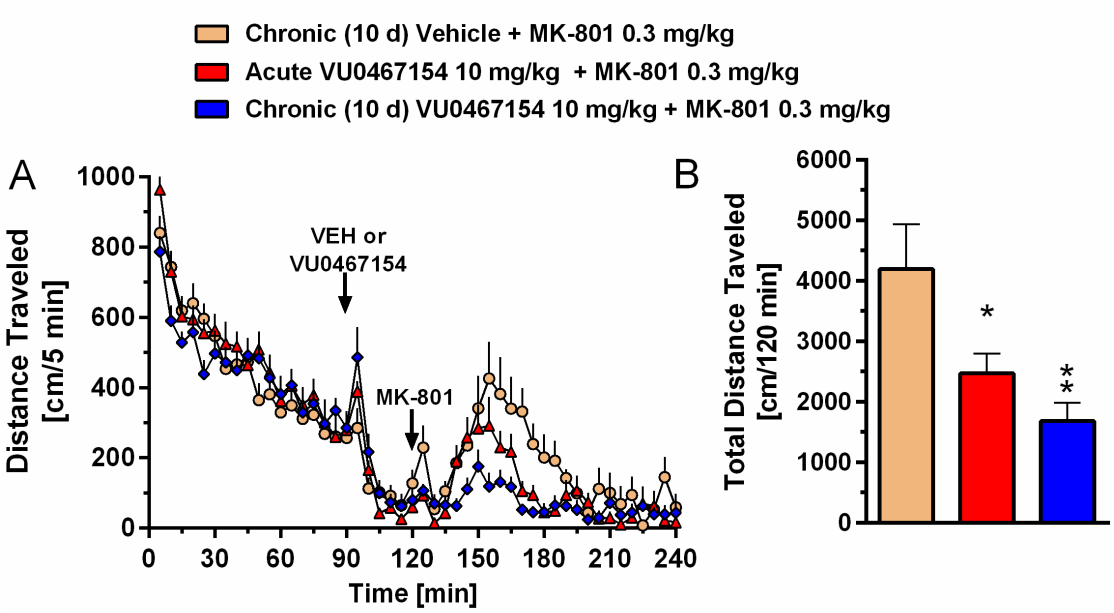


Figure 24. Repeated once-daily dosing of 10 mg/kg VU0467154 attenuates MK-801-induced hyperlocomotion on the 10th day of dosing in wildtype mice. A) Distance travelled in cm travelled/5 min bins, and B) total distance traveled in the 120 minutes following MK-801 administration; n=11-12/group per treatment group; * $p<0.05$, ** $p<0.01$ compared to vehicle+MK-801 treatment group. All data shown as means (± SEM).

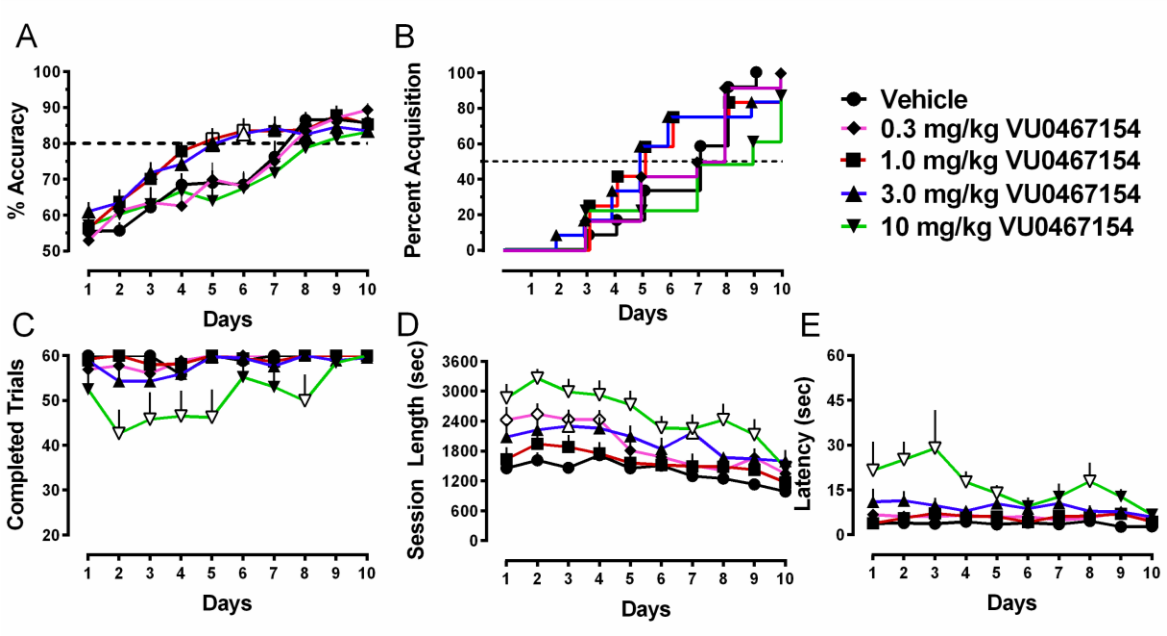


Figure 25. VU0467154 dose-dependently improves rate of acquisition of a pairwise discrimination task in mice when administered 60 minutes prior to each daily test session. A) Percent accuracy across the first 10 days of learning a PWD task. B) Percent of mice that reached acquisition criteria (>80% accuracy) across the 10 days of learning a PWD task. C) Total number of completed trials, D) session length, and E) average latency to respond for all correct trials, reward across 10 days of learning a PWD task; n=10-12 mice/group; open symbols, $p<0.05$ compared to vehicle-treated mice on the same day. All data shown as group average (± SEM).

	1 mg/kg		3 mg/kg		10 mg/kg	
	total	unbound	total	unbound	total	unbound
plasma [nM]	383.8 (268.8)	8.44 (5.9)	1239.7 (539.0)	27.3 (11.9)	3034.3 (1350.8)	66.8 (29.7)
brain [nM]	84.3 (0.5)	1.2 (0.01)	159.1 (62.4)	2.2 (0.9)	304.8 (145.5)	4.2 (2.0)
K_p $K_{p,\,uu}$		0.22 0.14		0.13 0.08		0.10 0.06

Table 3. *In vivo* plasma and brain concentrations following repeated dosing of VU0467154. Arithmetic mean (± SD) of total and calculated unbound plasma and brain concentrations of VU0467154 in wildtype mice 2.5 hr after intraperitoneal administration of 1, 3 or 10 mg/kg VU0467154 once daily for 14 days; n = 4−5/ group. K_p, $K_{p,uu}$, total and calculated unbound brain/plasma partition coefficients. Mouse plasma and brain unbound fractions, 0.022 and 0.014, respectively.

We also examined the number of completed trials, overall session length, average response latency on correct trials and average reinforcer retrieval latency to provide measures of motor function or motivation to respond that may indirectly influence learning. As shown in Figure 25C, there was a significant effect of dose ($F_{4,499}$=18.96, $p<0.0001$) and day ($F_{9,499}$=2.70, $p<0.01$) on number of trials completed. As shown in Figure 25D, there was a significant effect of dose ($F_{4,499}$=43.63, $p<0.0001$) and day ($F_{9,499}$=12.34, $p<0.0001$) on session length such that treatment with VU0467154 increased total session length (open symbols significantly different from vehicle, $p<0.05$). There was also a significant effect of dose ($F_{4,498}$=1.88, $p<0.0001$) on correct response latency (Figure 25E), such that 10 mg/kg VU0467154 significantly increased latency. Similarly, there was a significant effect of dose ($F_{4,497}$=7.27, $p<0.0001$) and day ($F_{9,497}$=2.12, $p<0.05$) on reinforcer retrieval latency such that 10 mg/kg increased latency, while lower doses did not affect retrieval latency (data not shown). Significant effects of post-hoc comparisons between respective treatment group compared to vehicle-treated mice within each day are denoted by open symbols ($p<0.05$). In general, effects were dose-dependent and driven by the 10 mg/kg treatment group, suggesting that at the highest dose tested, nonselective disruptive effects on motor output or motivation may contribute to lack of cognitive enhancing effects; however, tolerance developed to these disruptive effects across the 10 days.

This study represents the first characterization of the effects of repeated administration of an M_4 PAM on measures of cognition. To confirm that effects on memory were not influenced by pharmacokinetic factors (e.g. cytochrome P450 autoinduction or steady-state accumulation), a subset of mice from this pairwise

discrimination study was administered VU0467154 for an additional 4 days (total 14 days of dosing), followed by measurement of plasma and brain levels of VU0467154 at 2.5 hr (after the last dose). As shown in Table 3, repeated dosing of 1, 3, and 10 mg/kg VU0467154 produced 1.2, 2.2, and 4.2 nM calculated unbound brain concentrations. The 2.5 hr post-final dosing time point was chosen to correspond with the previously reported time point for assessment of plasma and brain concentrations following a single administration of 1, 3, and 10 mg/kg VU0467154 in mice, which resulted in 1.0, 2.2, and 5.6 nM calculated unbound brain concentrations (Bubser et al., 2014). Thus, 10 day of repeated dosing produced plasma/brain concentrations similar to those observed from a single dose of VU0467154, consistent with the once daily dosing interval (24 hr) encompassing ≥5 half-lives, and thereby precluding steady-state accumulation.

Experiment 3. Assessment of the effects of repeated (10 days) dosing of VU0467154 on rate of learning and acquisition of a pairwise discrimination task in M_4 KO mice. To determine whether enhancement of the rate of learning a pairwise discrimination task by VU0467154 was mediated through potentiation of M_4 mAChRs, we administered vehicle or 3 mg/kg VU0467154 60 minutes before test sessions for 10 consecutive days in M_4 KO mice. As shown in Figure 26A, over the 10-day period, there was a significant increase in percent accuracy ($F_{9,135}$=16.76; $p<0.0001$), but no effect of treatment. There were no days on which VU0467154-treated mice differed from vehicle-treated mice (all $p>0.05$). As shown in Figure 26B by day 10, 30% of the vehicle-treated mice and 60% of the VU0467154-treated mice acquired the discrimination within 10 days. 2 of the total 19 mice that did not improve above 55% accuracy across the 10 days were excluded from overall data analysis. There was a significant effect of day

($F_{9,135}$=32.16, $p<0.0001$) on session length (Figure 26C) and significant effects of dose ($F_{1,149}$=11.32, $p<0.01$) and day ($F_{9,149}$=2.19, $p<0.05$) on correct response latency (Figure 26D); both measures decreased across sessions and were not different between treatment groups. There were no differences in number of trials completed or reinforcer retrieval latency between groups.

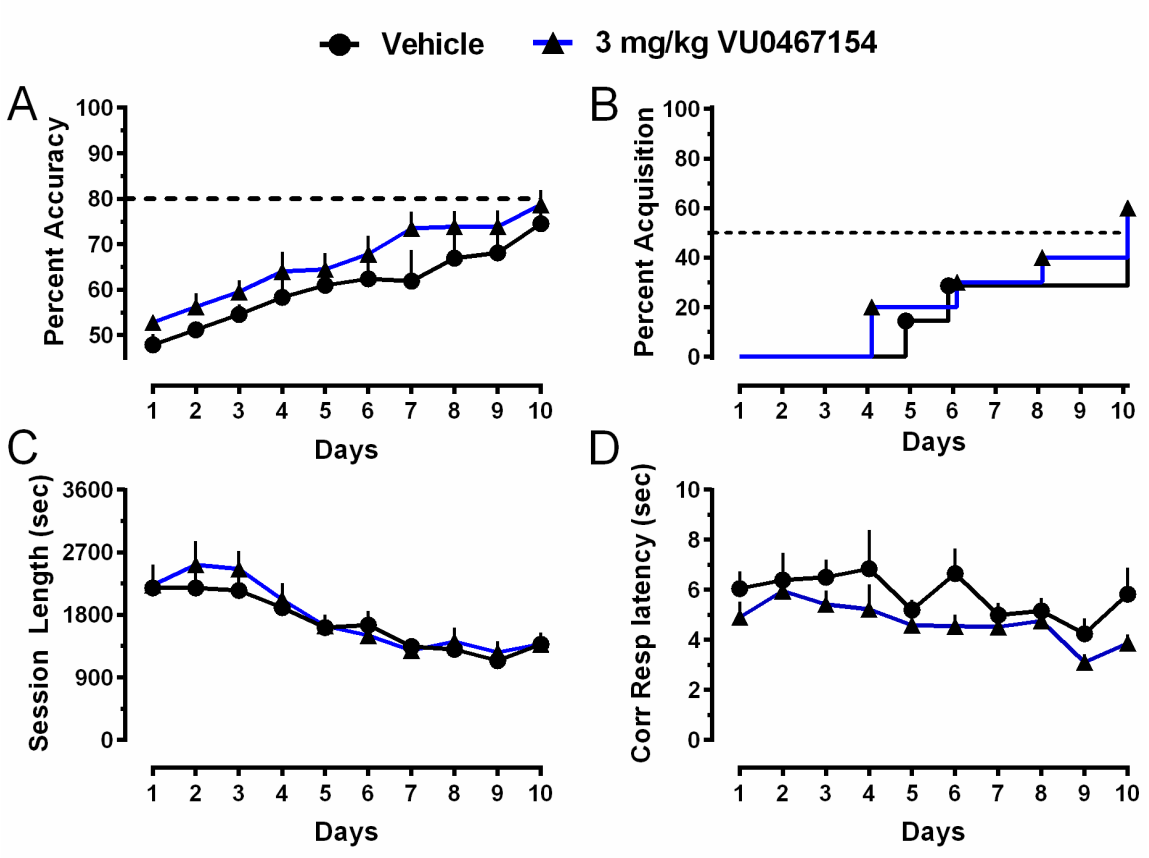


Figure 26. VU0467154 does not improve rate of acquisition of a pairwise discrimination task in M_4 KO mice when administered 60 minutes prior to each daily test session. A) Percent accuracy across the first 10 days of learning a PWD task. B) Percent of mice that acquired (>80% accuracy) across the 10 days of learning a PWD task. C) Average total session length, D) average latency to respond for all correct trials across 10 days of learning a PWD task; n=7-10 mice/group. All data shown as group average (± SEM).

Experiment 4. Assessment of the effects of repeated (10 days) dosing of VU0467154 on acquisition versus consolidation of a pairwise discrimination task in wildtype mice. To determine whether enhancement of the rate of learning a pairwise discrimination task by VU0467154 was through acquisition or consolidation of memory, we administered 1 mg/kg VU0467154 60 minutes before or immediately after each session for 10 consecutive days. As shown in Figure 27A, over the 10-day period, there was a significant increase in percent accuracy ($F_{9,205}$=26.11; p<0.0001), and a significant effect of dose/time of dosing ($F_{2,205}$=18.08; p<0.0001). Dunnett's post-hoc analysis revealed that mice treated with 1 mg/kg VU0467154 before daily sessions demonstrated a significant improvement in rate of learning, as shown by significantly higher percent accuracy on days 3-5. Mice treated with 1 mg/kg VU0467154 after each session demonstrated a significant improvement on day 3, compared to the vehicle-treated wildtype mice (all p<0.05); there were no significant differences between groups treated with VU0467154. As shown in Figure 27B, 50% of mice dosed with VU0467154 before and after daily sessions acquired the discrimination on days 3 and 4, respectively, compared to 6 days in the vehicle-treated group.

There was a significant effect of dose ($F_{2,205}$=13.64, p<0.0001) and day ($F_{9,205}$=2.76, p<0.01) on correct response latency (Figure 27C) and a significant effect of dose ($F_{2,204}$=8.83, p<0.001) and day ($F_{9,204}$=3.10, p<0.05) on reinforcer retrieval latency (Figure 27D). Correct response latency was higher in mice treated with VU0467154 before the session on day 3 compared to post-session VU0467154 or vehicle-treated groups (p<0.05). Reinforcer retrieval latency was higher on day 3 in mice administered VU0467154 before the session compared to vehicle (p<0.05) (not shown). However,

similar to data in Experiment 2 (Figure 25), significant effects on latency were driven by decreases across the 10-day period. There were no differences in number of trials completed or overall session length between groups.

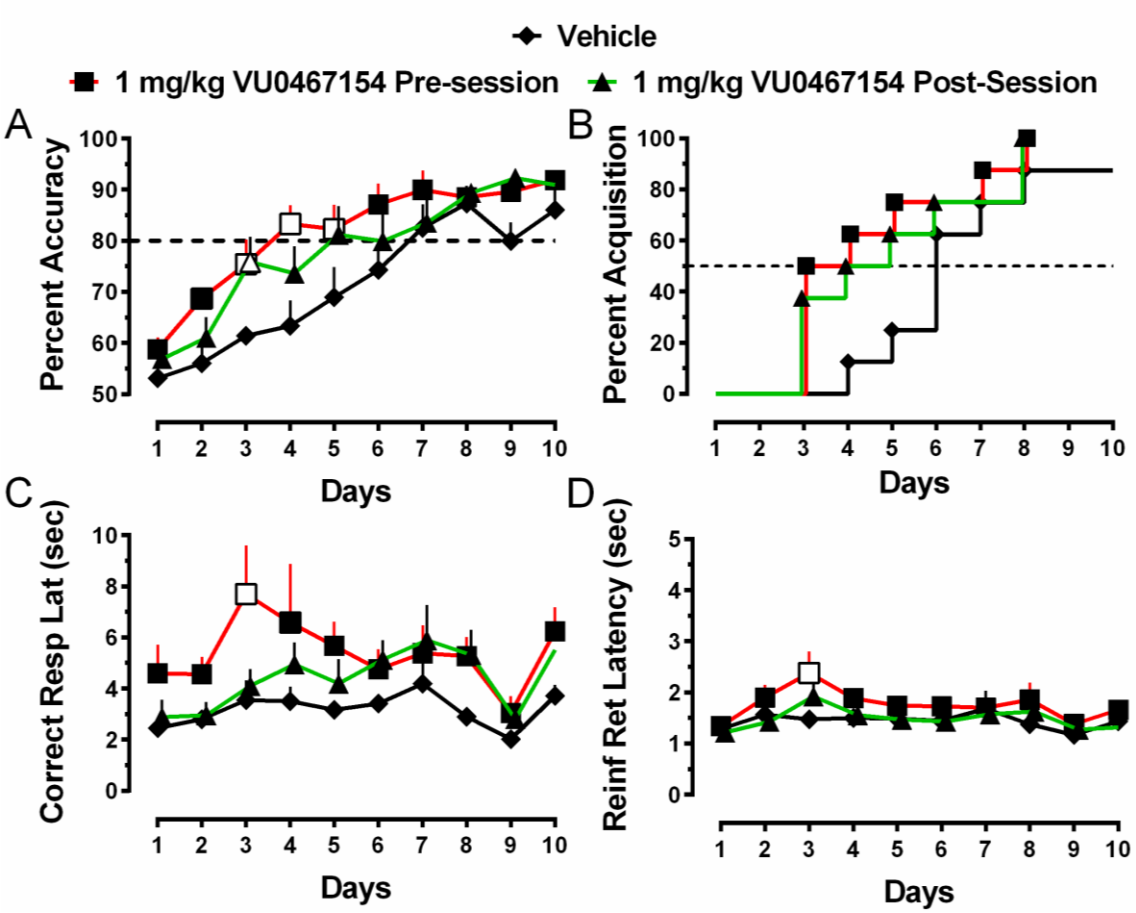


Figure 27. VU0467154 improves rate of acquisition of a pairwise discrimination task in wildtype mice (A, B) when administered 60 minutes prior to (red squares) or immediately following (green triangles) each daily test session. A) Percent accuracy across the first 10 days of learning a PWD task. B) Percent of mice that acquired (>80% accuracy) across the 10 days of learning a PWD task. C) Average latency to respond for all correct trials, D) and average latency to retrieve the reinforcer after each correct trial across 10 days of learning a PWD task; n=8 mice/group; open symbols, $p<0.05$ compared to vehicle-treated mice on the same day. All data shown as group average (± SEM).

Discussion

Selective activation of M_4 mAChRs represents an important novel mechanism for the potential treatment of multiple symptom clusters associated with schizophrenia, including positive symptoms and cognitive impairments. Given the dearth of M_4-selective ligands with suitable pharmacokinetic properties for *in vivo* use until recently, all previous preclinical studies have focused on evaluations of efficacy using acute dosing paradigms. The present studies provide the first demonstration that there is no development of tolerance to the antipsychotic-like activity and cognitive enhancing effects of M_4 PAMs after 10 days of repeated dosing. These studies confirm and extend accumulating evidence supporting future development of selective M_4 PAMs for the treatment of positive symptoms and cognitive deficits observed in various neuropsychiatric disorders, such as schizophrenia.

The current validation of M_4 PAM efficacy under conditions of repeated dosing provides a greater degree of translatability to support future clinical dosing approaches. Previous reports suggested a lack of cytochrome P450 induction by VU0467154 and that little or no accumulation at steady-state would occur from a once daily administration schedule. To confirm this, the brain and plasma concentrations of VU0467154 after 14 days of chronic dosing were measured as a control and found to be comparable to the levels (and brain:plasma distribution ratios) observed in previously reported studies by our group after acute administration of VU0467154 (see Table 3 from, Bubser et al., 2014). These pharmacokinetic findings confirmed that the observed efficacy with VU0467154 after repeated administration was not due to changes in exposure levels resulting from drug accumulation or from potential alterations in the metabolism of

VU0467154 through cytochrome P450 induction after chronic dosing.

The present findings also provide the first report of improved cognitive performance in a preclinical learning and memory task over multiple days through selective potentiation of M_4 mAChRs with repeated dosing of VU0467154. These findings with VU0467154 build on several previously reported studies that examined the acute effects of M_4 mAChR potentiation on cognition. In particular, the M_4 PAM VU0152100 enhanced performance in a version of an object recognition task following a 5 min delay (Galloway et al., 2014), and VU0467154 enhanced cue-mediated conditioned freezing following a 24-hr delay, two classic preclinical models of working and long-term memory, respectively (Bubser et al., 2014). VU0467154 also blocked MK-801-induced impairments on a contextual conditioned freezing task following a 24-hr delay and impairments on a pairwise discrimination task following acquisition (Bubser et al., 2014). By assessing alterations in the acquisition of pairwise discrimination task induced by VU0467154 over multiple days, we controlled for several sources of variability often associated with acute cognition studies, including individual differences and fluctuations in baseline performance, or introduction of a cognitive disruptor to artificially increase signal window to examine cognitive enhancing effects. As shown in the dose-response determination (Exp 2) and follow-up study (Exp 4), VU0467154 significantly improved rates of learning and acquisition by 2-3 days in both experiments, demonstrating reproducibility of a robust improvement on memory with chronic dosing. Importantly, these improvements in cognitive performance were absent in the M_4 KO mice, indicating that the effects of VU0467154 are mediated through actions at M_4 mAChRs.

An additional advantage for using operant cognition paradigms is the ability to

examine multiple dependent variables that can influence cognitive performance. For example, consistent with many cognition studies (Bentley et al., 2011), VU0467154 engendered an "inverted-U" dose response curve such that administration of 10 mg/kg did not improve cognition. This dose decreased the number of trials completed and increased total session duration, suggesting that 10 mg/kg VU0467154 indirectly influenced the outcome of cognitive performance (e.g. motoric, motivational effects). It is important to note that the present cognitive enhancing effects occur at dose ranges providing calculated unbound concentrations below the *in vitro* EC_{50} (~18 nM). Interestingly, the dose-exposure range producing cognition-enhancing effects overlaps with the low end of the range in which we have previously demonstrated antipsychotic-like effects in preclinical models of the positive symptoms of schizophrenia (Bubser et al., 2014). Two explanations for the differing dose ranges between these effects include treatment with the M_4 PAM alone versus reversing pharmacological disruptions, and divergent circuitry (e.g. mesolimbic versus mesocortical) underlying these processes as well as likely differences in M_4 receptor expression levels and receptor reserve that may influence the effects of VU0467154.

Understanding the potential mechanism(s) through which potentiation of M_4 activity by VU0467154 improves performance in the pairwise discrimination task holds potential promise for advancement of novel treatment approaches for the cognitive impairments in schizophrenia. Currently available typical and atypical APDs provide little, if any, therapeutic effects for the cognitive deficits observed in individuals with schizophrenia, and, in many cases, may actually further exacerbate cognitive disruptions (Price et al., 2014; Miyamoto et al., 2012). M_4 mAChRs are expressed throughout limbic

and cortical brain regions, including the striatum, hippocampus, and cortex, (Levey et al., 1991; Hersch & Levey, 1995; Rouse et al., 1999) and can function as autoreceptors in the striatum and midbrain (Zhang et al., 2002; Tzavara et al., 2004), as well as postsynaptic modulatory receptors in the striatum, neocortex, and hippocampus (Levey et al., 1991; Zang & Creese, 1997). For example, potentiation of M_4 mAChRs can selectively potentiate mAChR-mediated reductions in glutamatergic, but not GABAergic, neurotransmission in hippocampal neurons (Shirey et al., 2008), suggesting a possible key role for M_4 in regulating hippocampal function that may mediate the improved acquisition of the present learning and memory task observed with VU0467154.

Alternatively, effects of M_4 mAChR potentiation on enhancing memory, the only cognitive domain examined to date, may in part be mediated through mechanisms of consolidation versus specific memory encoding or acquisition. While enhanced muscarinic cholinergic function is critical for acquisition of new memory formation, previous preclinical and clinical studies have demonstrated that decreased cholinergic tone is critical for memory consolidation, particularly sleep-dependent memory consolidation (Gais & Born, 2004; Hasselmo & Sarter, 2011; Gais & Born, 2004). For example, scopolamine, a nonselective mAChR antagonist, has shown differential effects on memory using a conditioned freezing paradigm depending on the time of dosing relative to the training conditions. When administered prior to training, scopolamine disrupts memory retention 24 hr later (affecting acquisition of memory), yet when administered after the training period, facilitates memory retention, presumably through enhancing memory consolidation (Rasch et al., 2006; Winters et al., 2006). Declarative memory, one of the most disrupted cognitive domains in schizophrenia (Green et al.,

2000), is improved following slow wave-rich sleep compared to rapid eye movement-rich sleep in healthy subjects (Plihal & Born, 1997). Moreover, declarative memory is improved by increasing slow wave activity during slow wave sleep via transcranial direct current stimulation in patients with schizophrenia (Goder et al., 2013). Furthermore, we have recently shown that, in healthy rats, VU0467154 dose-dependently increases the ratio of slow wave sleep to rapid eye movement sleep (Gould et al., 2016).

In the present studies, we specifically examined potential M_4-mediated effects on memory consolidation versus acquisition in the pairwise discrimination task by administering 1 mg/kg VU0467154 either one hr prior to or immediately following test sessions. Following this dosing paradigm, mice receiving VU0467154 prior to each session would have peak concentrations of VU0467154 in the brain at the initiation of the session (T_{max} = 1 hr based on previous mouse IP PK studies). Given the moderate elimination half-life in this species (4.7 hr), these mice still possess pharmacologically relevant concentrations of VU0467154 in the brain for several hours after the session. Mice are nocturnal, and spend a large percentage of time sleeping during light periods. Since these studies were conducted during the light phase, mice presumably cycled in and out of sleep after each daily test session, while relevant concentrations of VU0467154 were present in the brain. Thus, VU0467154 may be affecting memory acquisition (during task performance) as well as consolidation (period directly after task performance thought to last ~1-6 hrs). However, in mice dosed immediately after the session, VU0467154 only affected memory consolidation. Importantly, results observed with dosing after each session were not quite as robust as results observed with dosing prior to each session, suggesting potential effects on both memory acquisition and consolidation.

While not yet available, confirmation of these effects using an equipotent M_4 PAM possessing a shorter elimination half-life, or administering an M_4 selective antagonist immediately after test sessions, will be important for future studies. Collectively, these data provide compelling evidence that selective potentiation of M_4 mAChRs enhances learning and memory, in part through memory consolidation. Ongoing studies are currently investigating the impact of selective potentiation of M_4 mAChRs by VU0467154 on additional cognitive domains, including attention and executive function that are reported to be impaired in patients with schizophrenia.

CHAPTER V

SELECTIVE POTENTIATION OF M_1 MUSCARINIC ACETYLCHOLINE RECEPTORS AMELIORATES ELECTROPHYSIOLOGIC AND BEHAVIORAL DEFICITS IN A GENETIC MODEL OF NMDAR HYPOFUNCTION

Introduction

Multiple lines of evidence suggest that disruptions in the normal signaling of the *N*-methyl-D-aspartate subtype of the glutamate receptor (NMDAR) may underlie many of the symptoms associated with schizophrenia, particularly the cognitive impairments(Balu, 2016; Kantrowitz & Javitt, 2010; Kantrowitz & Javitt, 2010). Previous studies have demonstrated that acute or chronic administration of noncompetitive NMDAR antagonists, including phencyclidine (PCP) and ketamine, induce psychotic-like symptoms and cognitive impairments in animals and healthy individuals and exacerbate symptoms in patients with schizophrenia (see review by (Balu, 2016; Krystal et al., 1994)). Recent copy number variation analysis studies have identified numerous *de novo* mutations in genes encoding the NMDAR and other proteins within the glutamatergic postsynaptic density associated with increased risk of schizophrenia (Fromer et al., 2014; Kirov et al., 2012; Purcell et al., 2014). Postmortem studies have also revealed reductions in the mRNA and protein expression of the NR1 subunit of the NMDAR in the prefrontal cortex (PFC) of individuals with schizophrenia (Catts et al., 2016). Moreover, studies using genetic models with deletion or knockdown (KD) of the GluN1 subunit of the NMDAR, either globally or constitutively in parvalbumin inhibitory neurons, cortical and hippocampal interneurons, or cortical pyramidal neurons have reported abnormalities in various physiologic measures,

including changes in gamma band oscillations, and impairments in social and cognitive performance similar to many of the symptoms observed in schizophrenia (Belforte et al., 2010; Jadi et al., 2016; Tatard-Leitman et al., 2015; Mohn et al., 1999; Milenkovic et al., 2014; Gregory et al., 2013). Taken together, targeting abnormalities in NMDAR function represents a critical strategy for the treatment of the complex symptoms associated with schizophrenia.

Development of ligands that selectively target the M_1 muscarinic acetylcholine receptor (mAChR) subtype represents an important approach for modulating NMDAR function and could lead to potential therapeutic interventions for schizophrenia (Jones et al., 2012), The M_1 mAChR subtype is one of the five different mAChR subtypes, termed M_1-M_5 (Bonner et al., 1987; Bonner et al., 1988), which are G-protein-coupled receptors (GPCRs) activated by the neurotransmitter acetylcholine (ACh). M_1 is highly expressed across many forebrain regions implicated in the pathophysiology of schizophrenia, including the striatal complex, all layers of the cortex, and postsynaptically on the dendrites and cell bodies of hippocampal granule cells and pyramidal neurons (Levey et al., 1995; Levey et al., 1991; Marino et al., 1998; Rouse et al., 1998; Rouse et al., 1999). Postmortem studies have reported that M_1 mAChR expression is reduced in the PFC, hippocampus, and other forebrain regions of a subset of patients with schizophrenia (Dean et al., 2002; Gibbons et al., 2013; Scarr et al., 2013). More importantly, previous studies have shown that M_1 is both physically and functionally coupled to NMDARs and activation of M_1 potentiates NMDAR currents in cortical and hippocampal pyramidal cells, increases excitatory postsynaptic currents in PFC neurons, and enhances performance in several preclinical learning and memory tasks (Gould et al., 2015;

Ghoshal et al., 2016; Jones et al., 2012; Shirey et al., 2009; Sur et al., 2003; Jones et al., 2008; Digby et al., 2012). Conversely, M_1 knockout (KO) mice have impaired performance in medial prefrontal cortex (PFC)-dependent cognitive tasks and reduced hippocampal long-term potentiation (LTP) (Anagnostaras et al., 2003; Gould et al., 2015; Bartko et al., 2011; Miyakawa et al., 2001). While clinical studies with selective M_1 ligands have been limited, studies with the M_1/M_4-preferring mAChR agonist xanomeline produced robust efficacy in reversing psychotic symptoms and behavioral disturbances, with some modest effects on cognitive impairments, in both schizophrenia and Alzheimer's disease patient populations (Bodick et al., 1997a; Bodick et al., 1997b; Shekhar et al., 2008). Unfortunately, xanomeline, similar to other orthosteric mAChR agonists, failed to progress in clinical development due to dose-limiting adverse effects from nonselective activation of other mAChR subtypes (Bymaster et al., 2002).

Over the last decade, we and others have developed subtype-selective M_1 mAChR ligands; these compounds do not target the orthosteric binding site of acetylcholine (ACh) that is highly conserved across all five mAChR subtypes, but rather act at more topographically distinct allosteric sites (Digby et al., 2012; Jones et al., 2008; Jones et al., 2012). In particular, our group and others have now reported the discovery and extensive optimization of multiple novel chemical series of highly selective M_1 positive allosteric modulators (PAMs) which enhance the efficacy and/or affinity of the endogenous neurotransmitter Ach (Bridges et al., 2010; Bridges et al., 2010; Bridges et al., 2010; Marlo et al., 2009; Melancon et al., 2013; Shirey et al., 2009; Ma et al., 2009; Mistry et al., 2013; Uslaner et al., 2013). Earlier generation M_1 PAMs served as important tool compounds for the *in vitro* characterization of the molecular and slice-based physiologic

activity of selective M_1 potentiation, but provided limited utility for animal studies due to poor central penetration (Kuduk et al., 2010; Kuduk et al., 2010; Kuduk et al., 2011; Shirey et al., 2009; Thomsen et al., 2012). Using several second generation M_1 PAMs with more favorable pharmacokinetic (PK) properties for *in vivo* dosing, selective potentiation of M_1 has been reported to reverse pharmacologically induced deficits and/or improve normal performance on many measures of learning, memory and top-down prefrontal processing in rodents and nonhuman primates (Ma et al., 2009; Gould et al., 2015; Chambon et al., 2012; Chambon et al., 2011; Galloway et al., 2014; Uslaner et al., 2013; Ghoshal et al., 2016; Shirey et al., 2009; Reid et al., 2011; Poslusney et al., 2013; Panarese et al., 2016). For example, the M_1 PAM BQCA robustly enhanced the synaptic excitation of pyramidal cells in the PFC and improved PFC-mediated cognitive functions, including performance in attentional set shift tasks (Shirey et al., 2009). More recently, we reported the discovery of the selective M_1 PAM VU0453595 that enabled further characterization of the role of M_1 on PFC-mediated synaptic plasticity, cognitive function and social interaction. Specifically, VU0453595 reversed chronic PCP-induced disruptions in muscarinic long term depression (mLTD) in the PFC and corresponding deficits in novel object and social recognition tasks (Ghoshal et al., 2016). However, to date the progress in understanding the role of M_1 modulation in PFC-mediated physiology and behavior relevant to schizophrenia has been achieved using only acute and chronic NMDAR antagonist challenge approaches in adult animals.

Recent development of several genetic models of reduced NMDAR activity have provided important *in vivo* systems for studying changes in relevant cortical and limbic circuitry comparable to those observed in schizophrenia (see (Balu, 2016; Ferris et al.,

2014; Milenkovic et al., 2014; Ramsey, 2009; Belforte et al., 2010; Jadi et al., 2016; Tatard-Leitman et al., 2015)). While the null mutation of *grin1*, the gene encoding the NR1 subunit of NMDARs, is lethal, a hypomorphic or partial loss-of-function mutation of *grin1* results in an NR1 subunit knockdown (NR1 KD) mouse that is still viable, but displays only 10-15% of the wild-type levels of NMDARs and exhibits severe behavioral and physiologic impairments, many of which are consistent with those induced by acute and/or chronic NMDAR antagonist challenge (Ramsey, 2009). We and others have reported that NR1 KD mice display increased locomotor and stereotypic behaviors that can be reduced with antipsychotic drugs and a novel metabotropic glutamate receptor subtype 5 ($mGlu_5$) PAM, as well as severe impairments in social interactions and cognition (Ramsey, 2009; Gregory et al., 2013). In the present studies, we specifically evaluated PFC-mediated physiologic and cognitive functions in NR1 KD mice. We first used slice-based extracellular field potential recordings and found deficient muscarinic agonist-induced LTD in layer V of the PFC in NR1 KD mice. Using *in vivo* electrophysiological recordings in awake, freely moving NR1 KD mice, we report excessive pyramidal neuron firing in layer V PFC neurons. NR1 KD mice were also impaired in their responses in two preclinical models of PFC-mediated learning and memory, the novel object recognition and cue-mediated fear conditioning tasks. Importantly, we report the ability of the novel and highly potent M_1 PAM VU6004256 to reverse these selective PFC-mediated abnormalities in synaptic plasticity, *in vivo* physiology, and behavior in NR1 KD mice.

Methods

Chemicals. VU6004256 (4,6-difluoro-*N*-(1*S*,2*S*)-2-hydroxycyclohexyl-1-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridine-3-yl)methyl)-1*H*-indole-3-carboxamide) and VU0453595 (Ghoshal et al., 2016; Panarese et al., 2016) were synthesized in-house and dissolved in DMSO for slice physiology studies or 10% Tween 80 in sterile water for *in vivo* studies. Carbachol (Sigma, St. Louis, MO) stocks were prepared in water and all other compound stocks were prepared in DMSO. For field recording experiments, all drugs were diluted in artificial cerebrospinal fluid (ACSF) (0.1% DMSO final conc.) and bath applied. All chemicals were obtained from Sigma (St. Louis, MO).

Animal Care and Housing.

All *in vivo* studies were carried out using age-matched adult male NR1 KD mice with a C57B1/6J and 129X1/SvJ crossed background and wild-type littermate controls (described previously by (Mohn et al., 1999; Milenkovic et al., 2014; Gregory et al., 2013)). 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 Animal Care and Use Committees of Vanderbilt University and University of Toronto, 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.

Novel Object Recognition Task

Mice were placed in a dark blue novel object recognition arena (32 X 32 X 40 cm) and allowed to habituate for 10 minutes. On the following day, mice were injected with either vehicle (10% tween 80) or VU6004256 (3 mg/kg or 10 mg/kg, i.p.) and returned to their home cages for 5 minutes. Mice were then placed in the novel object recognition arenas with 2 identical objects for 10 minutes. Following the initial exposure, mice were placed in their home cages for 1 hour. Upon completion of the 1 hour period, mice were returned to the arenas and one of the familiar objects was replaced with a novel object. Mice were recorded for 10 minutes and data were scored by 2 observers blinded to experimental conditions. Recognition index scores were calculated using the following equation: (seconds spent exploring novel object – seconds spent exploring familiar object)/total time exploring either object.

Locomotor Activity Studies in Mice

Spontaneous locomotor activity was assessed in 8-10 week old wild-type and NR1 KD mice. Animals were placed in an open field system (OFA-510, MedAssociates, St. Albans, VT) with three 16 × 16 arrays of infrared photobeams as described previously (Byun et al., 2014). Mice were allowed to habituate in their home cage for one hour prior to the start of the experiment. Following the habituation period, mice were injected with either vehicle (10% tween 80, i.p.) or a dose of VU6004256 (1 mg/kg or 10 mg/kg, i.p.) and placed directly into the open field chambers. Spontaneous locomotor activity was recorded for 90 minutes. The time course of drug-induced changes in spontaneous locomotor activity is expressed as distance traveled (cm) per 5 minute bin for the 90

minute session. Total activity data represent the sum of all 5 minute bins for the recording session. Data are represented as mean ± SEM and were analyzed using two-way ANOVA with Bonferroni's post hoc test.

In Vivo Electrophysiology

The method was a modified version of that described in (Walker et al., 2008). Mice anesthetized with isoflurane were secured on a stereotaxic apparatus and a craniotomy was performed at AP(+1.7 mm) ML(+0.5 mm). The electrode bundle was composed of eight 25 μm Formvar-insulated stainless-steel wires for recording single units and two 50 μm uninsulated stainless-steel ground wires, one of which served as a depth reference electrode. The bundle length was 2.5 mm, allowing for recording of prelimbic mPFC pyramidal cells when the array is fixed to the skull with dental acrylic. Mice are allowed to recover 5-7 days before entering the recording arena. All recordings took place between 12pm and 4pm. A wire harness attached to a headstage containing unity gain amplifiers was secured to the electrode array and mice were allowed to explore the chamber for fifteen minutes prior to the initiation of baseline recording. Animals were placed in a chamber equipped with a Faraday cage and attached to an electric commutator which allowed for unrestricted movement during the *in vivo* recording.

Neuronal discharges were acquired by the Multichannel Acquisition Processor (MAP) system following preamplification (Plexon Inc, Dallas, TX). To detect spiking activity, signals were bandpass filtered (154 Hz to 8.8 kHz) and digitized at a rate of 40 kHz. After establishing a voltage threshold ≥2.5 times background noise, waveforms were continuously collected and principal component analysis was used to discriminate

putative pyramidal neurons from fast-spiking interneurons. All recording sessions lasted for 75 minutes. Baseline firing rate was assessed during the final 5 minutes of the baseline collection period (30 minutes), and the effects of vehicle (10% tween 80, i.p.) or VU6004256 (10 mg/kg , i.p.) were assessed during the final 5 minutes of the drug treatment period (45 minutes). Data are expressed as mean frequency during each period or as a percent of baseline and significance is determined using a paired t-test.

Extracellular Field Potential Recordings

8-10 week old male NR1 KD or wild-type littermate control mice were anesthetized with isofluorane, and the brains were removed and submerged in ice-cold cutting solution (in mM: 230 sucrose, 2.5 KCl, 8 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 10 D-glucose, 26 $NaHCO_3$). Coronal slices containing the prelimbic prefrontal cortex were cut at 400 μm using a compresstome (Precisionary Instruments, Greenville, NC). Slices were transferred to a holding chamber containing NMDG-HEPES recovery solution (in mM: 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$, 12 *N*-acetyl-L-cysteine, pH 7.3, <310 mOsm) for 15 minutes at 32 ºC. Slices were then transferred to a room temperature holding chamber for at least 1 hour containing ACSF (in mM: 126 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$) supplemented with 600 μM sodium ascorbate for slice viability. All buffers were continuously bubbled with 95% O_2/5% CO_2. Subsequently, slices were transferred to a 32 ºC submersion recording chamber where they were perfused with ACSF at a rate of 2 mL/min. Borosilicate glass electrodes were pulled using a Flaming/Brown micropipette

puller (Sutter Instruments) and had a resistance of 2-4 MΩ when filled with ACSF. Sampled data was analyzed offline using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA). The slopes from three sequential sweeps were averaged. All slopes calculated were normalized to the average slope calculated during the pre-drug period (percent of baseline). Data were digitized using a Multiclamp 700B, Digidata 1322A, and pClamp 10 software (Molecular Devices). Input-output curves were generated to determine the stimulation intensity that produced 50-60% of the maximum response before each experiment, which was used as the baseline stimulation. A minimum of 10 min stable baseline was recorded before application of any drugs. 50 and 10 μM carbachol was applied for 10 min to induce saturated and threshold form of muscarinic LTD in the prefrontal cortex. M_1 PAM VU6004256 was treated alone for 10 min immediately followed by co-application with carbachol. Carbachol (Sigma, St. Louis, MO) stocks were prepared in water and all other compound stocks were prepared in DMSO. All drugs were diluted in ACSF (0.1% DMSO final conc.) and bath applied.

Fear Conditioning

Contextual- and cued-mediated conditioned freezing. 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 (MedAssociates, Allentown, NJ). To assess ability of NR1 KD mice to learn both contextual and cue-mediated associative learning, we conducted studies across a 72 h period, with initial training occurring on Day 1, testing of contextual conditioned freezing on day 2, and testing cue-mediated conditioned freezing on Day 3. Wildtype and NR1

KD mice were handled for three days prior to study initiation. On the conditioning day, mice were habituated for 1 h in an anteroom. Mice were then placed into the chamber - scented with 1.0 mL 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) co-terminating with a shock (0.7mA, 1 s) with an inter-tone interval of 60 s, followed by a 90 s interval without any stimuli. Approximately 24 h after conditioning (Day 2), mice were placed in the anteroom for 1 h and then exposed to the same conditioning context (identical environment and conditioning chamber including tactile, light, and odor cues). Freezing behavior, defined as motionless posture, excluding respiratory movements, was measured in the absence of any tone or shock stimuli for 8 min. After this 8 min test mice were returned to their homecage. Approximately 48 h after conditioning (Day 3), mice were returned to the anteroom and habituated under infrared light for 1 h. 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 the conditioning day (e.g. presentation of the 4 tones) 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 ± S.E.M. and analyzed by one-way ANOVA followed by Bonferroni's test.

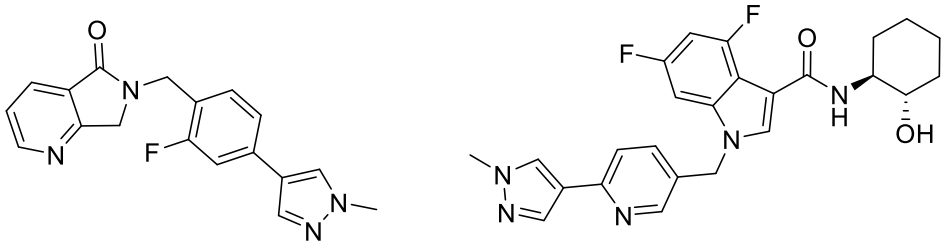
Cue-Dependent Fear Conditioning. Studies were conducted using conditioning chambers in sound attenuating cubicles equipped with a stainless steel grid floor for

shock delivery and a video camera for recording freezing behavior as previously described (MedAssociates, Allentown NJ, see (Bubser et al., 2014)). Wild-type and NR1 KD mice, 8-10 weeks old, were handled for 3 days prior to conditioning and injected with saline for 1 day prior to commencement of the study. On the conditioning day, mice were habituated for 1 h in an anteroom adjacent to the test room. Mice were pretreated with vehicle (10% Tween 80 in sterile water i.p.) or a dose of VU6004256 (10 mg/kg i.p.) 5 min 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 the conditioning day but without the shock stimuli. Freezing behavior was measured in the absence of any shock stimuli for 8 min. Data are presented as means ± SEM and analyzed by one-way ANOVA followed by Bonferroni's test.

Results

VU6004256 is a highly potent M_1 Positive Allosteric Modulator with optimized pharmacokinetic properties relative to the previously reported M_1 PAM VU0453595.

As shown in Figure 28, the structure of the novel M_1 PAM 4,6-difluoro-*N*-(1*S*,2*S*)-2-hydroxycyclohexyl-1-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridine-3-yl)methyl)-1*H*-indole-3-carboxamide **2** (VU6004256) is compared side-by-side with previously published M_1 PAM VU0453595, **1** (Ghoshal et al., 2016; Lindsley et al., 2014; Tarr et al., 2012). VU6004256 is a potent potentiator of mouse M_1 (EC_{50} = 155 nM, 88% ACh Max) and is ~20-fold more potent than VU0453595. In addition, VU6004256 displays mouse brain:plasma partitioning (K_p) coefficient of 4.84 and an unbound brain:plasma coefficient ($K_{p,uu}$) of 2.6. In addition to selectivity for M_1 ($M_{2\text{-}5}$ EC_{50}s >30 µM), VU6004256 also displayed no off-target pharmacology at over 68 GPCRs, ion channels and transporters in an ancillary pharmacology radioligand binding panel (% inhibition @ 10 µM <50%). Therefore, VU6004256 represents a more potent and centrally penetrant M_1 PAM compared to VU0453595 to explore efficacy in NR1 KD mice as a genetic model of NMDAR hypofunction.



VU0453595 (**1**)
mouse M_1 EC_{50} = 3.2 μM
75% ACh Max
mouse K_p = 0.13
mouse $K_{p,uu}$ = 1.1

VU6004256 (**2**)
mouse M_1 EC_{50} =155 nM
88% ACh Max
mouse K_p = 4.84
mouse $K_{p,uu}$ = 2.6

Figure 28. Structure, potency and pharmacokinetic properties of the novel M_1 PAM 4,6-difluoro-*N*-(1*S*,2*S*)-2-hydroxycyclohexyl-1-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridine-3-yl)methyl)-1*H*-indole-3-carboxamide (VU6004256) (right) in comparison with the previously published M_1 PAM VU0453595 (Left).

Deficits in Carbachol-Induced LTD Can Be Rescued by VU6004256.

Bath application of carbachol (50 μM) induced a robust muscarinic LTD response in layer V mPFC of wild-type mouse tissue (68.5% ± 7.0 of baseline; Figure 29A). This effect was dramatically blunted in NR1 KD mouse tissue (88.3% ± 5.1 of baseline; Figure 29B), but bath application of VU6004256 (1 μM) during the carbachol application resulted in normalization of the muscarinic LTD response (Figure 29C) to levels comparable to wild-type mouse tissue (65.3% ± 1.6, $H = 10.41$, $p < 0.01$; Figure 29D).

VU6004256 Attenuates Excessive Pyramidal Cell Firing in the Prefrontal Cortex of NR1 KD Mice.

In vivo electrophysiology recordings from the prelimbic cortex of awake, behaving mice revealed a significant increase in firing rate of pyramidal neurons in NR1 KD mice (1.44 Hz ± 0.079) compared to wild-type littermate controls (1.090 Hz ± 0.0603)($t_{141} = 3.544$, $p < 0.0005$; see Figure 30B). Mice received either vehicle or 10 mg/kg VU6004256 following 30 minutes of baseline recording (Figure 30A). Pyramidal cell firing rate was reduced in the NR1 KD animals following VU6004256 administration (85.3% ± 4.1, $t_{69} = 2.09$, $df = 69$, $p < 0.05$; Figure 30F), with no observed effect of vehicle dosing (105.8% ± 9.4; Figure 30F). Vehicle or VU6004256 administration did not affect firing rate in wild-type animals (101.8% ± 5.1 and 88.7% ± 7.7, respectively; Figure 30C). Comparison of raw firing rates using a paired *t*-test revealed a strong post-dose decrease in NR1 KD mice following VU6004256 administration ($t_{37} = 3.76$, $p < 0.001$; Figure 31H) but no effect in wild-type animals (Figure 30E).

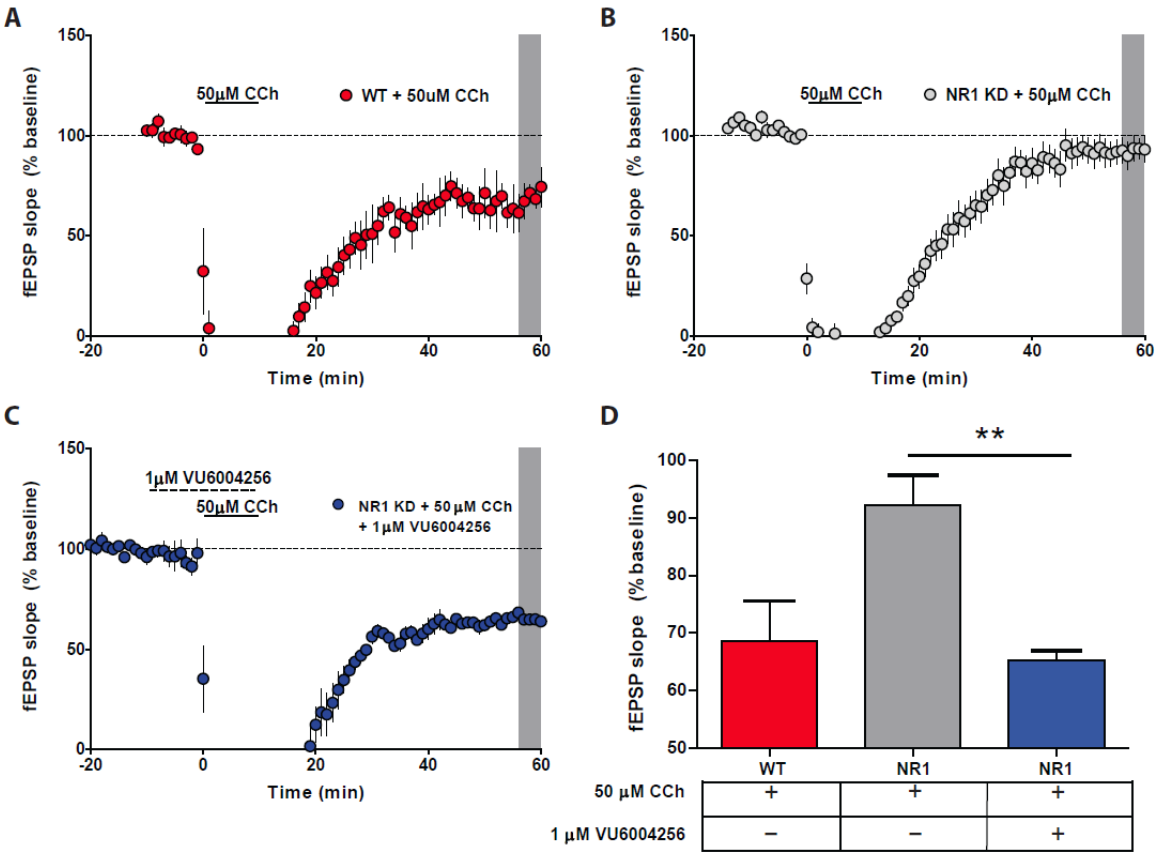


Figure 29. Muscarinic LTD is absent in NR1 KD animals but can be rescued by the M_1 PAM VU6004256. (A) CCh application (50 μM) induced LTD in layer V mPFC in WT mice (red circles, shaded gray area). (B) CCh-induced LTD is absent in NR1 KD animals, but treatment with VU6004256 (1 μM) before and during CCh application resulted in significant induction of LTD (blue circles, gray shaded area). (D) Graphical representation comparing minutes 55-60 from panels A-C. (**p = 0.0055, ANOVA, Kruskal-Wallis followed by Dunn's post hoc test).

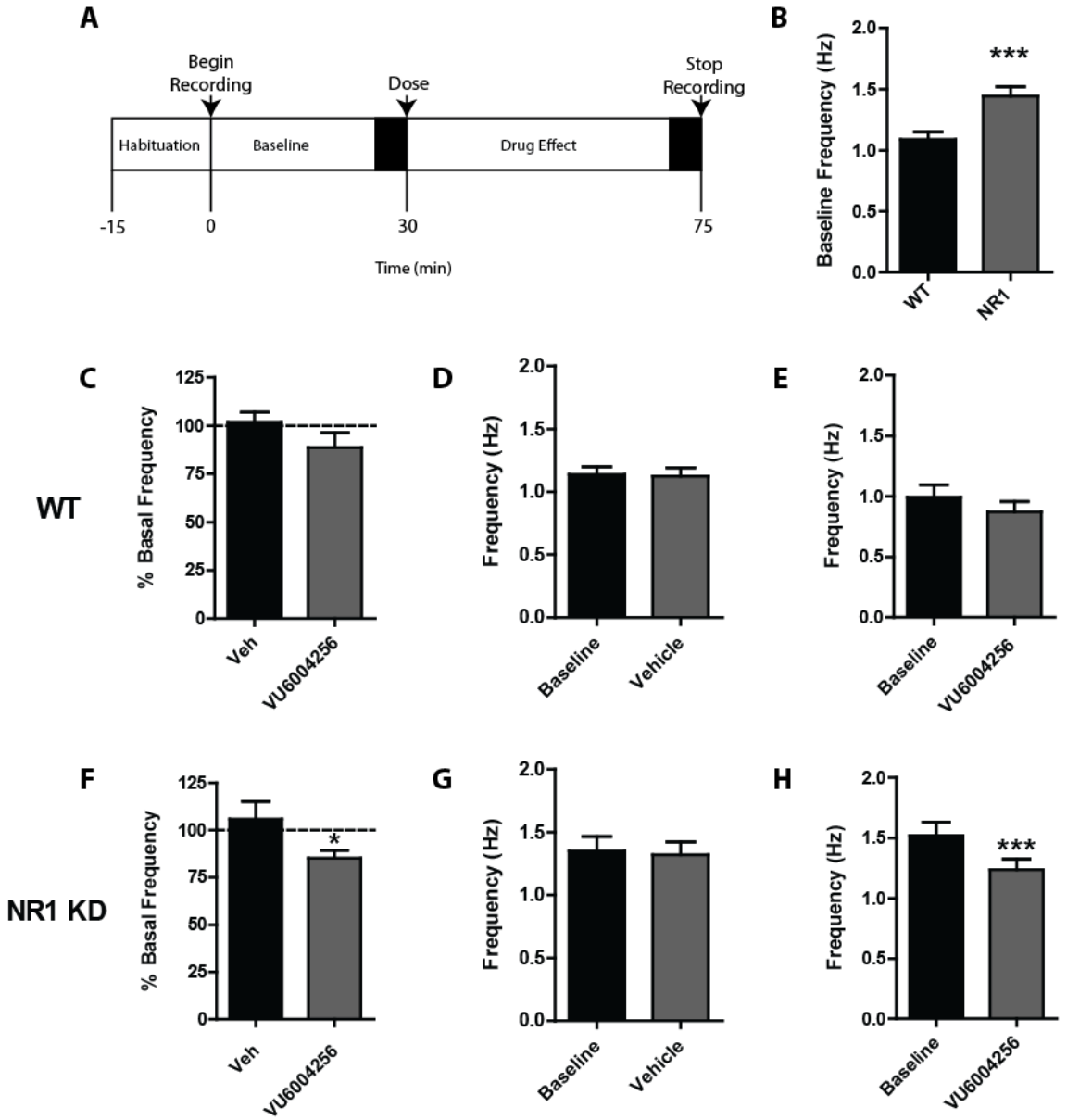


Figure 30. Comparison of WT and NR1 KD mouse pyramidal neuron firing rate in mPFC in awake, freely moving mice. (A) Recording paradigm with depiction of when firing rate averages were assessed (black boxes). (B) NR1 KD mice exhibit significantly increased firing rate compared to WT littermate controls. (C-E) VU6004256 had no effect on pyramidal cell firing rate WT littermate controls. (F-H) VU6004256 significantly reduced pyramidal cell firing rate in NR1 KD mice. Data in panels C and F represent changes in frequency compared to the baseline recording period prior to drug administration. (B, C and F) t-test, *$p < 0.05$. (D, E, G, and H) Paired t-test, ***$p < 0.001$. B: n=71-72 cells, C-H: n=33-41 cells.

VU6004256 Administration Improves Performance in Novel Object Recognition.

NR1 KD mice showed a marked, but statistically insignificant reduction in recognition index compared to their WT littermate controls (-0.0085 ± 0.0156 vs. 0.1150 ± 0.0563, respectively, Figure 31). Pretreatment with VU6004256 at either 3 mg/kg (0.1475 ± 0.0381) or 10 mg/kg (0.1662 ± 0.0401) significantly improved recognition index in NR1 KD mice ($F_{dose(1,44)}$ = 14.64, $p < 0.05$; $F_{genotype(2,44)}$ = 11.43, $p < 0.05$; $F_{interaction(2,44)}$ = 0.056, n.s.), while the top dose of 10 mg/kg (0.3125 ± 0.0552) also improved performance in WT littermate controls.

NR1 KD mice display robust learning and memory impairments.

Initial studies sought to characterize the ability of wild-type and NR1 KD mice to acquire contextual- (hippocampal-dependent) and cue-mediated (PFC/amygdala-dependent) fear conditioning responses. Findings measuring the effect of genotype on this response are reported in Figure 32A and B. Wild-type mice were able to acquire both contextual- and cue-mediated associative learning tasks (74.9 ± 6.0 and 17.4% ± 2.9, respectively). NR1 KD mice showed a lack of freezing in both paradigms (4.6% ± 1.8 and 1.5% ± 0.3, respectively) that was significantly lower than that of wild-type controls (context: t_{24} = 9.70, $p < 0.0001$; cue: t_{23} = 4.86). To specifically determine if potentiation of M_1 mAChRs would normalize PFC-mediated cognitive impairments, the effects of VU6004256 on cue-mediated fear conditioning were examined in wild-type and NR1 KD animals. NR1 KD mice displayed severe deficits in cue-mediated fear conditioning compared to their wild-type littermate controls (1.2% ± 0.7 and 41.9% ± 7.0, respectively; Figure 33). Pretreatment with 10 mg/kg VU6004256 5 minutes before being

placed in the chamber on the training day significantly improved performance on the test day in NR1 KD mice (12.3% ± 6.2, $t_{14} = 1.762$, $p < 0.05$).

To assess associative learning and behavioral flexibility using a translational touchscreen pairwise discrimination and reversal task, mice were trained to respond to one of two stimuli presented on the computer touchscreen. Responding on one stimulus (S+) resulted in reward delivery, whereas responding on the alternate stimuli (S-) terminated the trial. On the day following acquisition of the discrimination (>80% accuracy for 2 consecutive days), the rule for reinforcement would be reversed such that the previously non-reinforced stimulus is now reinforced and vice-versa. As seen in Figure 34, NR1 KD mice failed to show improvements in percent accuracy across the first 15 days of training. In contrast, WT mice showed increases in accuracy within several sessions (left) and 80% of the WT mice acquired the discrimination within 2 weeks (right) whereas not a single NR1 KD mouse reached 80% accuracy.

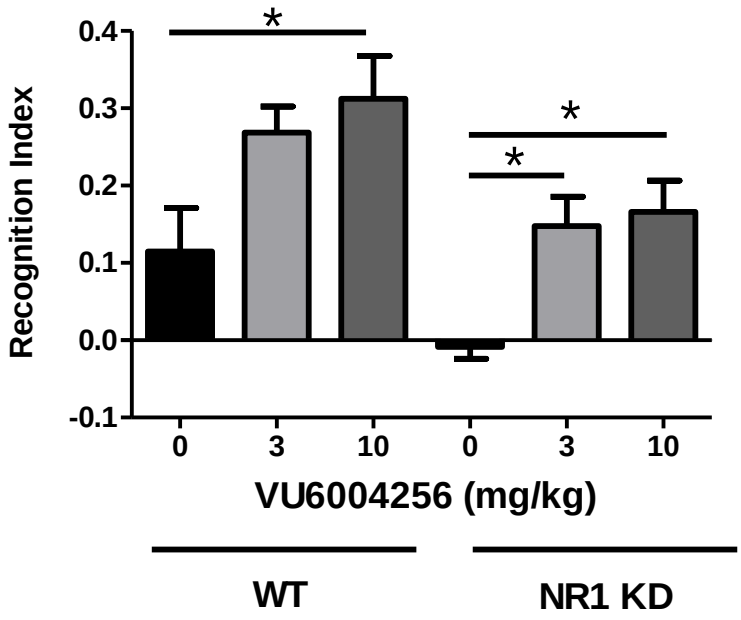


Figure 31. The novel object recognition deficit of NR1 KD mice is reversed by M_1 PAM administration. NR1 KD animals showed no preference for the novel object when treated with vehicle. Pretreatment with VU6004256 resulted in dose-dependent increases in recognition index. Acute treatment with 10 mg/kg VU6004256 in WT animals resulted in an increase in recognition index. (n=8-10/treatment group, * $p < 0.05$, 2-way ANOVA followed by Bonferroni's post hoc test).

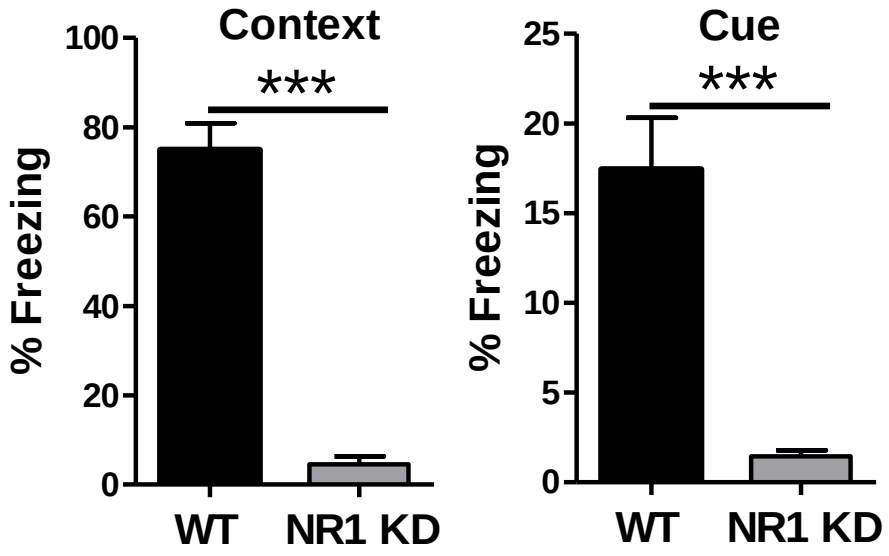


Figure 32. Comparison of NR1 KD and WT littermate control mice in context and cue-mediated fear conditioning. Left, NR1 KD mice displayed a significant reduction in time spent freezing compared to WT littermate controls. Right, Similar results were observed in the cue-mediated fear conditioning paradigm. (Unpaired t-test, ***$p < 0.0001$).

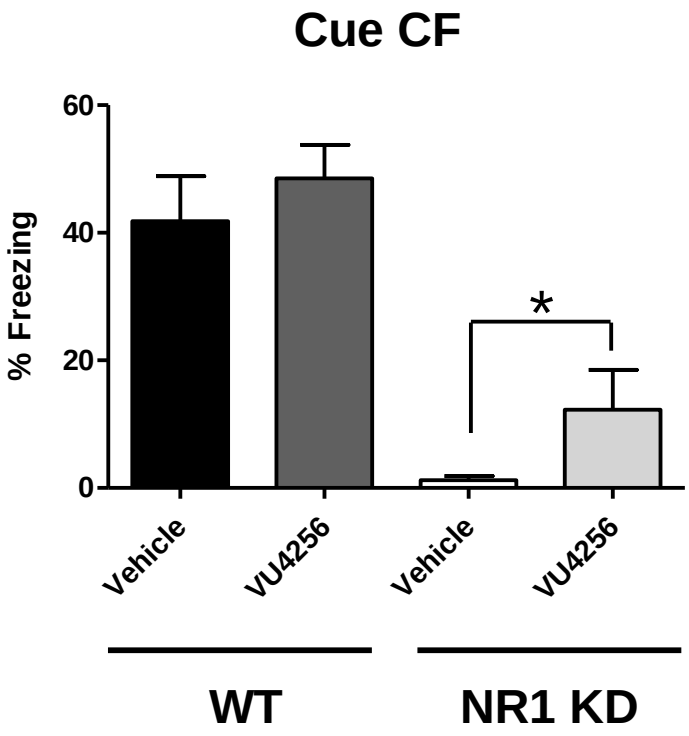


Figure 33. Cue-mediated conditioned freezing in WT and NR1 KD mice. Pretreatment with VU6004256 on training day significantly improved performance on test day in NR1 KD, but not WT mice. (n = 8-10/group, t-test, *p < 0.05).

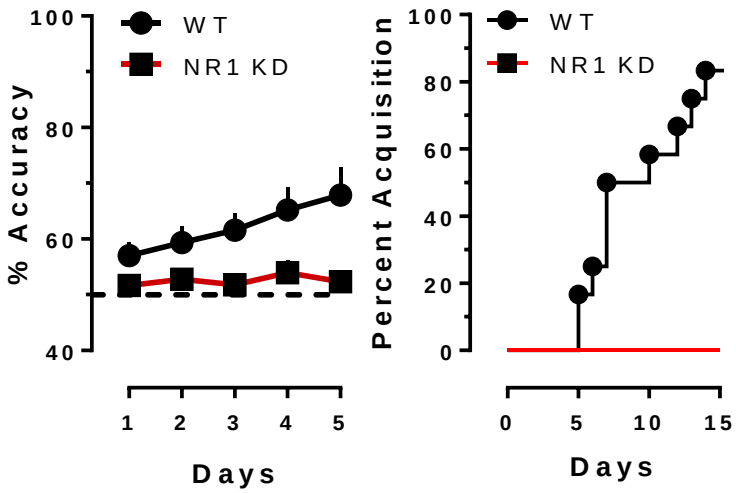


Figure 34. Training WT and NR1 KD mice in a model of associative learning and behavioral flexibility reveals cognitive deficits. NR1 KD mice do not learn the visual pairwise discrimination. In contrast, WT littermates acquire the pairwise discrimination task within 1 week of the initiation of training; n=12/group.

VU6004256 Reverses Excessive Locomotor Activity in NR1 KD Mice.

NR1 KD mice exhibit excessive spontaneous locomotor activity compared to their WT littermate controls ($F_{genotype(5, 39)} = 31.35$, $p < 0.0001$; see Figure 35B). Pretreatment with VU6004256 at 10 mg/kg produced a significant reduction in spontaneous locomotor activity that persisted for the duration of measurement ($F_{dose(5,612)} = 331.9$, $p < 0.001$; $F_{time(17,612)} = 6.101$, $p < 0.001$, $F_{interaction(85,612)} = 0.46$, n.s.; Figure 35A). No reduction in locomotor activity was observed at 1 mg/kg in NR1 KD mice. Additionally, pretreatment with VU6004256 at all doses did not alter spontaneous locomotor activity in WT littermate controls. Spontaneous locomotor activity was also measured in response to an additional M_1 PAM, VU0453595, with a distinct chemical scaffold (Figure 36). While NR1 mice exhibited a robust increase in spontaneous locomotor activity compared to WT littermate controls ($F_{dose(9,2400)} = 246.7$, $p < 0.001$; $F_{time(23,2400)} = 16.19$, $p < 0.001$, $F_{interaction(207,2400)} = 0.97$, n.s.), VU0453595 was unable to significantly alter this response, though there was a strong downward trend at the 1 mg/kg dose (Figure 36B).

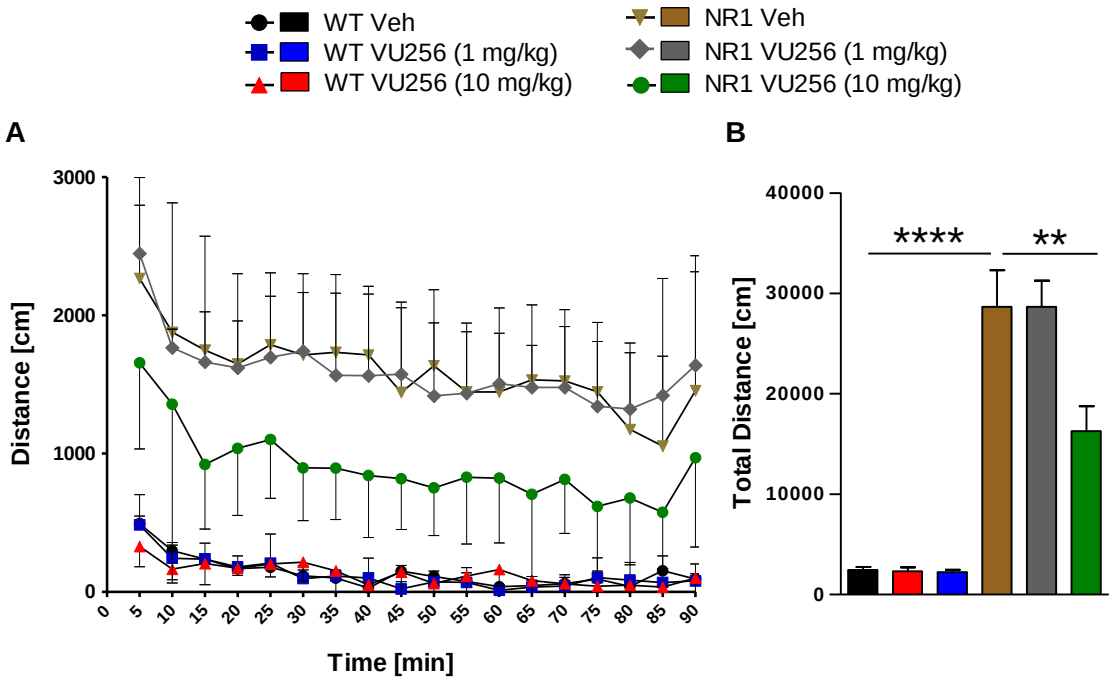


Figure 35. VU6004256 reduces excessive locomotor activity in NR1 KD mice. NR1 KD mice showed excessive locomotor activity when compared to WT littermate controls. Pretreatment with 1 mg/kg VU6004256 had no effect on locomotor activity in either WT or NR1 KD animals. Pretreatment with 10 mg/kg VU6004256 did not affect WT animals, but significantly reduced locomotor activity in NR1 KD mice. (n =7-9/treatment group, *p < 0.05, ANOVA followed by Bonferroni's post hoc test).

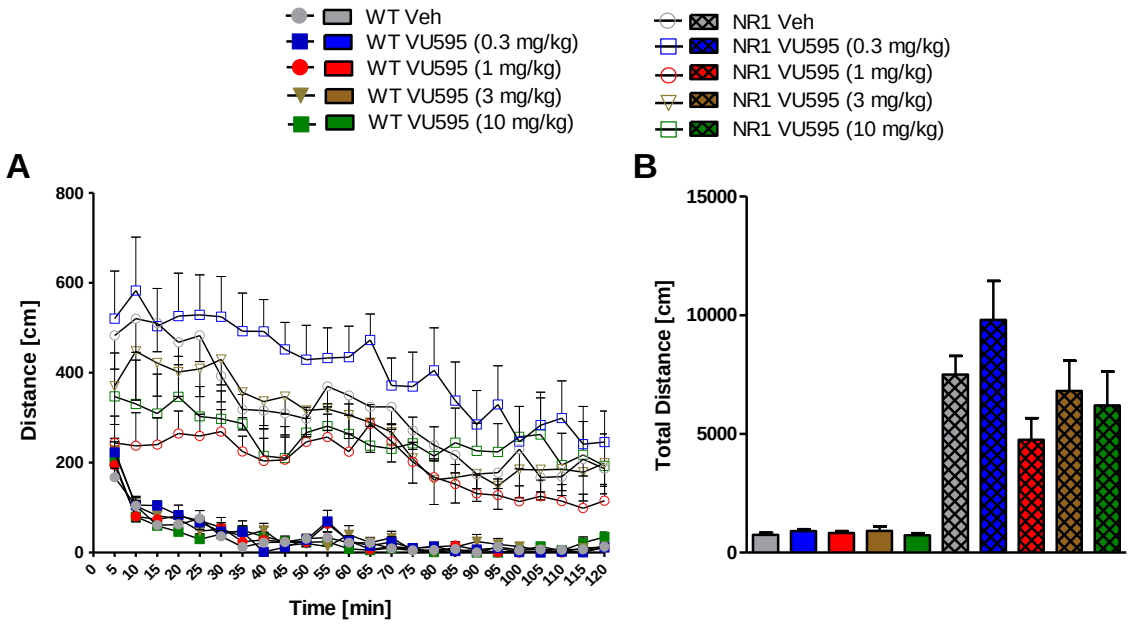


Figure 36. VU0453595 does not reduce hyperlocomotor activity in NR1 KD mice. Vehicle-treated NR1 KD mice showed excessive locomotor activity when compared to WT littermate controls. Pretreatment with VU0453595 (0.3-10 mg/kg) did not significantly affect locomotor response in NR1 KD or WT mice. (n =9-14/treatment group).

Discussion

As a close downstream signaling partner of the NMDAR, potentiation of the M_1 mAChR subtype represents an important indirect mechanism for the potential normalization of aberrant NMDAR signaling thought to underscore many of the symptoms observed in schizophrenia. In the present studies, we provide the first evidence of selective physiological dysfunction within the PFC and PFC-related cognitive deficits in a genetic model of global, constitutive knock down of the NR1 subunit of the NMDAR. More importantly, selective potentiation of M_1 using the optimized and highly potent M_1 PAM VU6004256 resulted in a restoration of these deficits in PFC-mediated synaptic plasticity, pyramidal cell firing, and corresponding measures of learning and memory.

Discovery of highly selective and potent M_1 PAMs allows for the investigation of antipsychotic-like effects that has been previously reported in preclinical and clinical studies with the M_1/M_4 preferring ligand, xanomeline.(Stanhope et al., 2001; Bodick et al., 1997a; Bodick et al., 1997b; Shekhar et al., 2008) While dose-dependent reversal of psychostimulant-induced increases in locomotor activity has been observed previously with the M_1 selective ligands, as demonstrated by the M_1 allosteric agonist TBPB and the M_1 PAM BQCA, much of the antipsychotic-like efficacy of xanomeline was attributed to M_4 activation.(Jones et al., 2008; Ma et al., 2009) In the current studies, we observed a significant reduction in elevated spontaneous locomotor activity of NR1 KD mice with a selective M_1 PAM in a genetic model of NMDAR hypofunction that is consistent with acute psychostimulant challenge models, which furthers the hypothesis that M_1 modulation in cortical and limbic regions may contribute to antipsychotic-like effects

observed with xanomeline. Interestingly, these effects are only observed with the more potent M_1 PAM, VU6004256 (EC_{50}=155 nM), and not with VU0453595 (EC_{50}=3.22 μM), suggesting VU6004256 may have efficacy in other behavioral models that less potent M_1 ligands do not. These findings provide valuable insight into the importance of developing more potent M_1 ligands to assess additional aspects of M_1-related antipsychotic-like efficacy.

Traditionally, acute or repeated dosing with a use-dependent open channel blocker of NMDAR is employed to produce NMDAR hypofunction in rodents that mimics neurochemical and behavioral changes observed in patients with schizophrenia.(Bubenikova-Valesova et al., 2008; Jentsch & Roth, 1999) There is a potential liability when evaluating M_1 PAMs in these models since M_1 is a closely associated signaling partner of NMDARs and selective activation of M_1 in cortical and hippocampal regions results in the downstream potentiation of NMDAR currents, which could exacerbate the effects of the NMDAR antagonist.(Marino et al., 1998) Because of the critical impact of NMDAR signaling in the modulation of normal cognition and neural circuitry hypothesized to be dysregulated in schizophrenia (Tsai & Coyle, 2002; Marino et al., 1998), ligands like the M_1 PAM VU6004256 that selectively potentiate the response of ACh at M_1 mAChRs may ameliorate many of the psychotic and cognitive impairments of schizophrenia through enhancement of NMDAR neurotransmission. Moreover, selective potentiation of M_1 by VU6004256 provides indirect modulation of NMDAR function, which may produce therapeutic effects without the potential high risk of excitotoxicity connected with direct agonist activity at the NMDAR. Importantly, these data provide the first evidence supporting antipsychotic-like effects with an M_1

PAM in a genetic model of NMDAR hypofunction that does not rely on pharmacologic manipulation to induce behavioral disruptions.

Previous reports indicate a crucial role for mAChRs in regulating synaptic plasticity in the PFC and suggest that deficits in cholinergic signaling may contribute to the electrophysiological and behavioral phenotypes associated with dysfunctional PFC plasticity. For example, non-selective activation of mAChRs is sufficient to convert transient depression into long-lasting LTD in the PFC following a sub-threshold LTD protocol in rats (Lopes-Aguiar et al., 2013). Importantly, acute co-application of an NMDAR antagonist blocked this response, suggesting an integral role of glutamatergic activation in muscarinic-mediated cortical LTD. The effects of mAChRs on this form of plasticity in the PFC were further characterized in studies utilizing M_1 knockout mice and the M_1 PAM VU0453595, revealing that muscarinic LTD is dependent on M_1 activation and can be potentiated by an M_1 PAM. Interestingly, synaptic depression was not observed following repeated NMDAR administration during early adolescent development, strengthening the importance of intact NMDA-M_1 receptor signaling in cortical synaptic plasticity.(Ghoshal et al., 2016)

Here we report the first electrophysiological analysis of spontaneous pyramidal cell activity in the layer V PFC of awake, free-moving NR1 KD mice in comparison with their wild-type littermate controls. Our findings revealed substantial information-processing deficits (Jackson et al., 2004) at the single-neuron level as denoted by a 25% increase in the firing rates in the NR1 KD mice. This disinhibition of PFC pyramidal cell firing in the NR1 KD mice is consistent with previous studies performed in rodents after acute administration of MK-801 or PCP (Jackson et al., 2004). At present, the underlying

mechanism(s) for this disinhibition of PFC activity in the NR1 KD mice remains unknown. However, based on NMDAR antagonist studies, one possible explanation may involve increased PFC activation resulting from disinhibition of hippocampal-PFC inputs (Jodo, 2013; Jodo et al., 2005; Kamiyama et al., 2011; Thomases et al., 2014), resulting in reduced LTD and enhanced LTP mechanisms. (Thomases et al., 2014; Kamiyama et al., 2011) This interpretation is further supported by the observation that the novel M_1 PAM VU6004256 decreased the firing rates in the NR1 KD mice. Alternatively, activation of M_1 is known to increase excitability in GABAergic interneurons of the PFC (Yi et al., 2014) through an NMDAR-independent mechanism; thus, another possible explanation for the effects of VU6004256 on pyramidal cell firing rates in NR1 KD mice may by through increased activity of GABAergic interneurons in the PFC, resulting in sustained reductions in PFC excitation. The ongoing development of cell type-specific NMDAR knockdown/knockout mice allows for more intricate examination of the role of NMDARs on local and projection neurons in the PFC and will be utilized by our group and others to elucidate the role of NMDAR hypofunction in the PFC. In future studies, it will also be important to further understand the relative contributions of M_1 modulation to both NMDAR-dependent and -independent mechanisms of PFC circuitry disrupted in the mice with global NR1 KD.

Genetic models of NMDAR hypofunction exhibit profound cognitive deficits as measured in radial arm maze performance, social and object recognition, and spatial and non-spatial memory tasks. (Belforte et al., 2010; Dzirasa et al., 2009; Rampon et al., 2000) Consistent with these findings, we observed robust deficits in context- and cue-mediated fear conditioning in NR1 KD mice, similar to the deficits encountered in NR2C

knockout mice.(Hillman et al., 2011) M_1 PAM administration improved deficits of NR1 KD mice in novel object recognition and cue-mediated fear conditioning, both of which are thought to heavily rely on intact PFC signaling. These data, taken together with recent reports that M_1 PAMs are efficacious in similar behavioral tasks performed in a pharmacologic model of NMDAR hypofunction (Ghoshal et al., 2016), suggest that M_1 potentiation may have broad utility across several animal models of schizophrenia.

Collectively, the current studies provide important novel insights into the role of M_1 modulation of PFC-mediated physiologic and cognitive functions under conditions of chronic NMDAR hypofunction. These findings also confirm and extend accumulating evidence for the broader therapeutic utility for M_1 PAMs in the treatment of affective and, more importantly, cognitive impairments observed in schizophrenia.

CHAPTER VI

DISCUSSION

In this work, we have demonstrated a broad therapeutic potential for the use of M_1 and M_4 PAMs as novel antipsychotic agents for the use in treating the positive symptoms, negative symptoms, and cognitive deficits observed in schizophrenia. In particular, we have shown the ability of an M_4 PAM to dose-dependently reverse psychostimulant-induced changes in locomotor activity, produced by either amphetamine or MK-801 administration. Importantly, these effects were absent in mice lacking functional M_4 receptors, suggesting the observed efficacy was due to activation of M_4 receptors in WT animals. In the case of amphetamine-induced changes in behavior, we were able to attribute the observed efficacy of an M_4 PAM to reductions in amphetamine-induced changes in dopamine release in the NAS and CP of rats. In addition, these changes in mesolimbic and mesostriatal circuitry were accompanied by overall reductions in CBV and regional connectivity in the retrosplenial and motor cortices, hippocampus and thalamus. These studies, taken together with a growing body of research discussed previously, suggest that M_4 PAMs reduce dopaminergic signaling and have a range of effects in rodent models predictive of antipsychotic-like activity. Importantly, these findings support a role for M_4 in the antipsychotic-like efficacy observed with the M_1/M_4-preferring ligand, xanomeline, and point to modulation of other brain circuits and neurotransmitter systems implicated in the cognitive deficits and negative symptoms observed in schizophrenia.

To address this question, further studies were carried out to determine the ability of an M_4 PAM to reverse NMDAR antagonist-induced changes in electrophysiological and behavioral deficits that are more closely associated with brain regions involved in the cognitive deficits and negative symptoms observed in schizophrenia. In these studies, we showed that an M_4 PAM could reverse 5-HT-evoked changes in glutamate release, as measured by sEPSC frequency in a slice-based electrophysiology paradigm, in layer V mPFC. The response to 5-HT has been attributed to activation of presynaptic 5-HT_{2A} receptors located presynaptically on glutamatergic thalamocortical terminals in the mPFC. Upon activation of these 5-HT_{2A} receptors, there is a dramatic increase in the frequency of spontaneous excitatory postsynaptic currents (EPSCs) measured in layer V pyramidal cells of the mPFC (Aghajanian & Marek, 1997; Aghajanian & Marek, 1999; Marek et al., 2001; Marek et al., 2000). Previous studies have demonstrated that atypical antipsychotics, including clozapine and olanzapine, inhibit this effect and reduce spontaneous EPSCs in the mPFC by direct antagonist activity at 5-HT_{2A} receptors on thalamocortical terminals. Additionally, others have demonstrated that the selective $mGlu_2$ PAM BINA and $mGlu_{2/3}$ agonists also blocked increases in the frequency of sEPSCs in layer V pyramidal cells of the PFC induced by activation of 5-HT_{2A} receptors on these presynaptic glutamatergic thalamocortical terminals in the PFC (Benneyworth et al., 2007). To confirm a role for M_4 in this circuit, a series of optogenetic experiments were performed, in which optically-evoked EPSCs were measured in pyramidal cells of the mPFC following viral-mediated gene transfer of CHR2 in MD thalamic neurons. These studies confirmed a role for M_4 modulation of this circuit, with decreases in CV analysis suggesting a presynaptic mechanism of action. Subsequent studies evaluating the

presence of M_4 transcript on MD thalamic projection neurons to layer V mPFC in rats, confirmed the presence of M_4 receptors in this circuit and validated the functional data observed in the slice electrophysiology experiments. The schematic depicted in Figure 37 shows the hypothesized expression of M_4 receptors in thalamocortical circuitry.

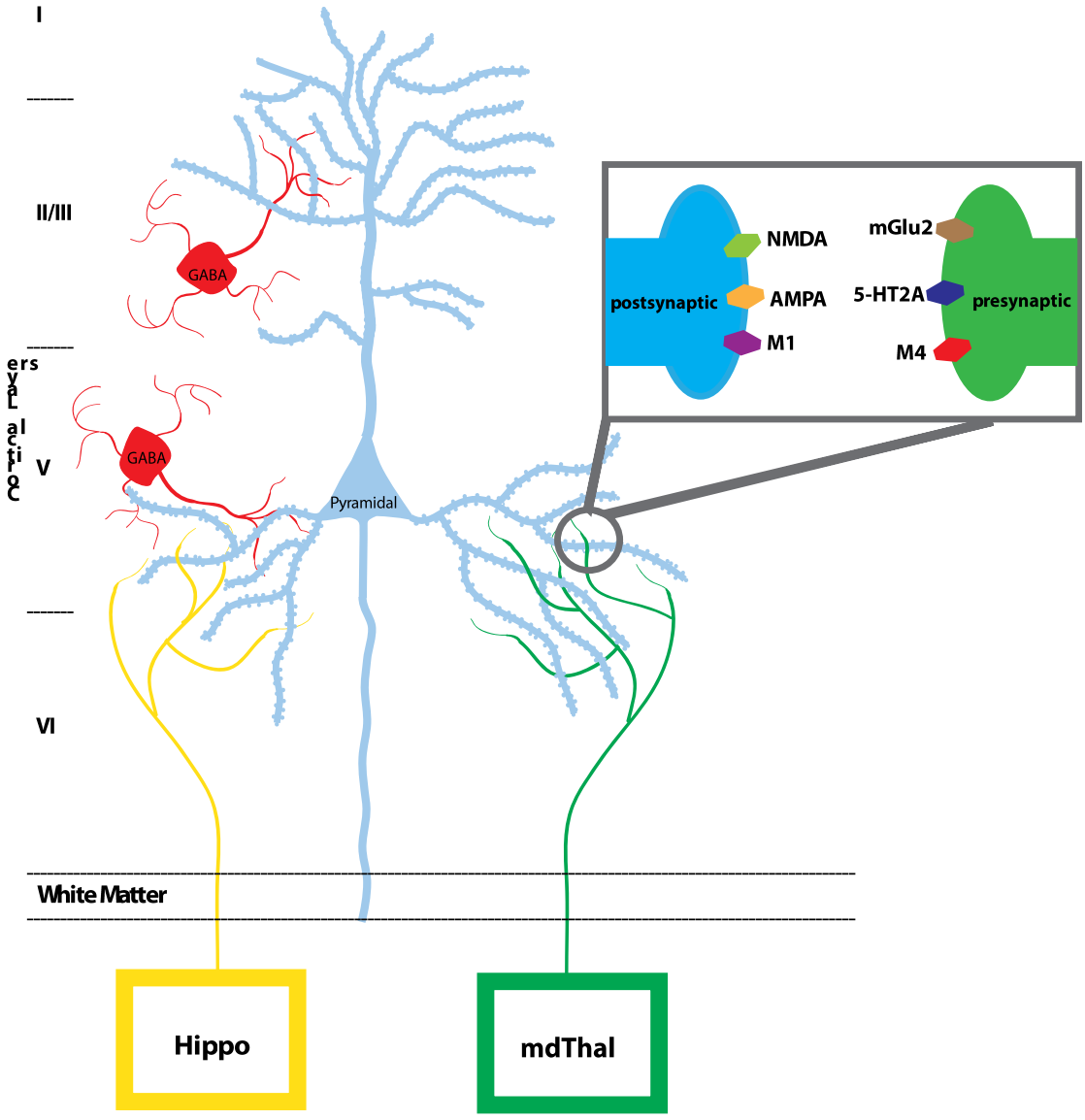


Figure 37. Model of M_4 mAChR expression in thalamocortical circuit.

To determine if *ex vivo* modulation of the PFC by M_4 PAMs translated into changes in cognitive performance, we first needed to confirm activity of the M_4 PAM *in vivo*. Based on our findings in whole cell electrophysiology we hypothesized MK-801 administration *in vivo* would result in dramatic changes in mPFC electrophysiological properties. As expected, MK-801 administration resulted in a significant increase in pyramidal cell firing rate in layer V mPFC pyramidal neurons, and pretreatment with an M_4 PAM was able to significantly block this response. These data suggest that M_4 PAMs are efficacious in modulating electrophysiological responses to psychostimulants *in vivo*, thus cognitive and behavioral tasks that are sensitive to disruptions in cortical circuitry via psychostimulants or hallucinogens may be rescued by M_4 PAM administration. To test this hypothesis we first performed behavioral assessment of the effect of M_4 PAM treatment on (±) DOI-induced head twitch response in mice. Interestingly, the M_4 PAM was as efficacious as the $mGlu_2$ PAM BINA at reversing the induction of head twitches, suggesting there was sufficient acetylcholine present for the PAM to function, and that there is sufficient M_4 expression in the cortex to elicit a change in behavior in response to M_4 PAM treatment. We then assessed the performance of WT mice in an operant-based spatial alternation task, a more complex behavioral assay that is thought to more heavily rely on normal function of PFC circuitry. Administration of an NMDAR antagonist resulted in severe deficits in performance, but pretreatment with an M_4 PAM was able to significantly reverse this response, resulting in performance well above chance responding. These data would suggest that M_4 PAMs are capable of modulating PFC circuitry that is implicated in the cognitive deficits observed in patients with schizophrenia.

To date, the effects of M_4 PAMs have been primarily evaluated in acute behavioral and physiological experiments, with little work performed to test the consequences of repeated dosing on behavior responses. To determine the role of M_4 in cognition, and to understand the effects of prolonged treatment, we examined the effects of repeated dosing with the M_4 PAM VU0467154 on learning a touchscreen-based visual pairwise discrimination task. First, we observed no difference is plasma or brain concentrations of drug following 10 days of repeated dosing, when compared to a single dose, suggesting there are no changes in absorption, distribution, metabolism, or excretion in response to repeated dosing. As expected, this translated to no loss of efficacy in reversing NMDAR antagonist-induced changes in locomotor activity. These studies are of great importance, as a loss of efficacy with repeated administration would preclude the use of M_4 PAMs in a clinical setting. Of equal importance, repeated administration of VU0467154 resulted in a significant increase in the rate of acquisition of the pairwise discrimination task. This was observed when VU0467154 was administered before and after the training session, suggesting effects on cognition may in part be through consolidation of memory. Together these data further support a role for M_4 mAChRs in learning and memory and in the potential treatment of multiple symptom clusters associated with schizophrenia.

Finally, in Chapter V we evaluated the effects of the novel and highly potent M_1 PAM VU6004256 in ameliorating selective PFC-mediated physiologic and cognitive abnormalities in a genetic mouse model of NMDAR hypofunction. Using slice-based extracellular field potential recordings, deficits in muscarinic agonist-induced LTD in layer V of the PFC in the NR1 KD mice were normalized with bath application of VU6004256. Systemic administration of VU6004256 also reduced excessive pyramidal

neuron firing in layer V PFC neurons in awake, freely moving NR1 KD mice. Moreover, selective potentiation of M_1 by VU6004256 reversed the performance impairments of NR1 KD mice observed in two preclinical models of PFC-mediated learning, specifically the novel object recognition and cue-mediated fear conditioning tasks. VU6004256 also produced a robust, dose-dependent reduction in the hyperlocomotor activity of NR1 KD mice. These novel findings provide further support for M_1 PAMs as a novel therapeutic approach for the PFC-mediated impairments in schizophrenia.

Taken together, these findings support a role for both M_1 and M_4 in the observed efficacy in preclinical and clinical studies with xanomeline, and should provide sufficient evidence for further development of selective muscarinic ligands for use as potential antipsychotic agents. Importantly, our findings suggest selective potentiators for either M_1 or M_4 can have beneficial effects when administered in animal models that are used to assess antipsychotic-like efficacy or cognitive enhancement. Previous hypotheses (Grunze et al., 1996)efficacy on measures of cognition were attributed to M_1 potentiation. Here, we show that while M_4 potentiation does indeed result in attenuation of positive-like symptoms, there is growing evidence to support the cognitive enhancing properties. This is observed in psychostimulant and hallucinogen-induced deficit models, as well as when the M_4 PAM is administered alone in WT animals (Bubser et al., 2014; Gould et al., 2016). Our data suggest one mechanism for this observed efficacy is through modulation of PFC circuitry that is known to be involved in higher cognitive processes. Interestingly, M_4 PAMs also reverse MK-801-induced hyperlocomotion, which does not result in elevated dopamine release in the mesolimbic and mesostriatal pathways,

suggesting that changes in locomotion in this assay may be the result of M_4 activation in other circuits.

Conversely, the cognitive enhancing effects with xanomeline have been previously attributed to activation of M_1 receptors. Here, we do show that M_1 PAMs are capable of modulating PFC circuitry that is known to be heavily involved in cognition, as measured by changes in *ex vivo* and *in vivo* electrophysiology studies. This resulted in changes in performance in PFC-mediated behavioral tasks, namely cue-mediated CF and novel object recognition. This is consistent with previous reports that evaluated the effects of an M_1 PAM in a sub-chronic NMDAR antagonist paradigm of NMDAR hypofunction (Ghoshal et al., 2016). These findings are also in accordance with previous findings from our lab, which reported an enhancement in the rate of learning pairwise discrimination in a top-down cognitive assay in response to daily administration of an M_1 PAM (Gould et al., 2015). Our most recent findings with a more optimized M_1 PAM, VU6004256, show the ability of potentiating M_1 receptors to have an antipsychotic-like profile, as was seen in locomotor reduction in NR1 KD mice. These findings do not support a strict adherence to the idea that M_4 receptors are only involved in attenuating the positive-like symptoms and M_1 receptors are only involved in improving cognition. Rather, these studies raise the possibility that potentiators of either M_1 or M_4, or both, could be exciting mechanisms to explore for the treatment of all three symptom domains observed in schizophrenia.

There have been huge advances in the development of selective, potent, and bioavailable muscarinic agents in the last decade. As reported here, VU6004256 is many-fold more potent and selective than other previously reported M_1 PAMs, and possesses

pharmacokinetic properties that make it amenable to i*n vivo* dosing. Likewise, VU0467154 represents a highly potent and selective M_4 PAM with excellent drug metabolism and pharmacokinetic properties that have been extensively evaluated in acute and chronic dosing paradigms. This raises, for the first time, the interesting possibility of combining doses of selective M_1 and M_4 PAMs for evaluation in complex behavior studies. Future studies should evaluate the effects of varying doses of each and perform isobologram analysis to predict efficacy based on the concept of dose equivalence. This will become important, as behaviorally ineffective doses of either M_1 or M_4 PAM may have profound effects when combined with each other. By performing studies of this nature, it will be possible to determine the relative contribution of M_1 and M_4 to the observed clinical efficacy of xanomeline, but also opens up the possibility of using lower doses of each, and could limit the potential for off-target activation of other receptors in the CNS and periphery.

Similar studies will need to be performed in conjunction with treatment with traditional antipsychotic medications, such as haloperidol, clozapine, and olanzapine. As mentioned several times previously in this work, current antipsychotics are fairly effective at controlling the positive symptoms associated with schizophrenia, but do little to curb the cognitive deficits and negative symptoms. As such, removing a patient from treatment that stably controls psychotic episodes may be untenable, or even considered unethical. This would limit the patient population to those that are being treated shortly after their first psychotic break. While there is evidence to suggest a muscarinic potentiator could be efficacious in this population, it restricts the scope of treatment to a small group of patients, and may not be predictive of efficacy in later stages of the

disorder. To address this, clinical trials need to be designed with combination therapy in mind.

However, there are several potential complexities when performing studies of this nature. First, many of the currently available antipsychotics have significant activity at some of, or all, of the mAChRs. For example, the prototypical antipsychotic drug, clozapine, has nanomolar affinity for all five cloned muscarinic receptors (Bolden et al., 1992), where it has been reported to be a potent antagonist of M_1 (Sur et al., 2003; Weiner et al., 2004; Zorn et al., 1994) and $M_{2/3/5}$ mAChRs (Bymaster et al., 1996; Michal et al., 1999). Contradicting reports have suggested clozapine is a partial agonist at $M_{1/2/4}$ mAChRs (Fritze & Elliger, 1995; Olianas et al., 1997; Zeng et al., 1997; Zorn et al., 1994). Adding to the complexity further, the primary metabolite of clozapine in rodent and human, *N*-desmethylclozapine, also has significant activity at mAChRs, specifically as a potent agonist at M_1 receptors (Aravagiri & Marder, 2001; Baldessarini et al., 1993; Balu et al., 2012; Sur et al., 2003; Weigmann et al., 1999; Weiner et al., 2004). In fact, *N*-desmethylclozapine has been shown to increase cortical acetylcholine and dopamine release *in vivo* via stimulation of M_1 muscarinic receptors, suggesting co-administration of an M_1 PAM could be a very efficient way to further increase dopamine levels in the cortex, leading to enhanced cognitive performance (Li et al., 2005). These types of interactions between compounds could obviously greatly impact studies that utilize co-administration of muscarinic potentiators, leading to changes in observed efficacy with either the antipsychotic or muscarinic ligands alone.

For example, there is a recent report by (Choy et al., 2016) that evaluated the efficacy of the M_1 PAM, BQCA, in combination with currently available typical and

atypical antipsychotics in rodent models of NMDAR hypofunction. In these studies, BQCA was administered alone or in combination with haloperidol, clozapine, or aripiprazole, and the ability to reverse MK-801-induced deficits in sensorimotor gating and spatial memory function, as measured by PPI and Y-maze. While administration of BQCA alone had no effects in either model, there were differing levels of efficacy in reversing the MK-801-induced deficits in PPI when combined with a sub-effective dose of each of the three antipsychotics. Interestingly, this observed efficacy did not translate to reversals with all three antipsychotics in the Y-maze task, where BQCA in combination with haloperidol could not reverse the deficits. These findings are very interesting given the reported M_1 receptor agonist activity of atypical, but not typical, antipsychotics. This study highlights the importance of thoroughly investigating the mAChR activity of currently approved antipsychotics, and the need to carefully design experiments that will utilize a co-administration design.

Also, it is important to note the possibility of adverse side effects developing with co-administration studies. For example, the recent characterization of a highly selective M_1 PAM, Compound 38 (PF-06767832), showed that despite lacking activity at $M_{2\text{-}5}$, there were still gastrointestinal and cardiovascular side effects observed (Davoren et al., 2016). These effects were not observed in rodent models, but when advanced into safety toxicology tests in dogs, adverse events such as diarrhea, emesis, and convulsions were observed. Interestingly, the plasma concentration when these adverse events were observed was approaching 1 µM, well above the reported EC_{50} of 60 nM. In fact, these plasma values, along with calculated unbound brain concentrations of approximately 330 nM, are at or slightly below the *in vitro* agonist EC_{50}. This suggests that M_1 activation,

either through agonist or PAM activity, contributes to the cholinergic liabilities that were previously attributed to activation of the M_2 and M_3 receptors, and that combination therapy with an antipsychotic that has known M_1 agonist activity could be contraindicated.

In the reports described in this work, we took advantage of a genetic model of NMDAR hypofunction that utilizes a constitutive knockdown of the NR1 subunit of the NMDA receptor. This leads to greatly reduced expression of functional NMDARs in key brain regions that are known to be involved with the symptoms of schizophrenia, namely the PFC, hippocampus, and striatum (Mohn et al., 1999). This is important to the understanding of the role of NMDAR function in psychiatric illnesses, such as schizophrenia, for several reasons. First, constitutive genetic knockdown of NMDARs from birth, and throughout neurodevelopment, better mimics the global glutamatergic hypofunction that is thought to be present in schizophrenia. As discussed in Chapter I, changes in glutamatergic signaling are evident in patients with schizophrenia, and changes in NMDAR expression and function around the time of adolescence are thought to contribute to the development of the disease (Olney & Farber, 1995; Tsai & Coyle, 2002). While there are recent developments in the creation of $NR2_{A-D}$ knockout and cell-type selective NR1 knockout mice that lack normal glutamatergic signaling from birth, these models have been primarily useful for delineating the role of NMDARs in specific circuits or brain regions, and their behavioral deficits are not as well aligned with those observed following NMDAR antagonist administration (Billingslea et al., 2014; Forrest et al., 1994; Halene et al., 2009; Hillman et al., 2011; Tatard-Leitman et al., 2015; von Engelhardt et al., 2008; Zhao et al., 2005). Additionally, global NR1 KD mice represent a

novel approach to evaluate the effects of life long NMDAR hypofunction without the use of acute or repeated dosing with an NMDAR antagonist. As discussed in Chapter V, one approach to treating the electrophysiological and behavioral deficits induced by NMDAR hypofunction is to potentiate NMDAR function. Direct stimulation of NMDARs has been shown to lead to excitotoxicity and seizure liability, however, there has been some success in clinical trials administering the co-agonist glycine (Heresco-Levy et al., 1996; Javitt et al., 1994; Leiderman et al., 1996). Our studies point to a novel mechanism of NMDAR potentiation through activation of M_1 mAChRs. These studies would not be possible without the use of genetic tools, like the NR1 KD mice, since pharmacologic hypofunction models traditionally use non-competitive, use-dependent antagonists that would not allow for NMDAR potentiation.

It is important to mention the potential disconnect between animal models of schizophrenia, such as NMDAR antagonist administration or genetic models of NMDAR hypofunction, and what is actually observed in patients with schizophrenia. It has been repeatedly demonstrated that NMDAR antagonist administration in humans and rodents leads to enhanced glutamatergic activity and increased firing rate of cortical neurons, primarily through decreases in activity of GABAergic interneurons (Anticevic et al., 2015; Homayoun & Moghaddam, 2007). This fits with data suggesting that GABAergic interneurons are 10-fold more sensitive to NMDAR antagonists than pyramidal neurons, presumably through increased NMDAR-dependent signaling and membrane properties that result in more open NMDA channels (Grunze et al., 1996). While this state can recapitulate many of the behavioral symptoms observed in schizophrenia, it is in stark contrast to the vast majority of clinical reports that routinely find hypofrontality, rather

than hyperfrontality, in patients with schizophrenia (Andreasen et al., 1997; Andreasen et al., 1992; Buchsbaum et al., 1990; Hazlett et al., 2000; Ragland et al., 2007; Tamminga et al., 1992; Volz et al., 1999). One potential explanation for this involves a time-dependent neurobiological evolution of the disease, in which excessive signaling in the cortical regions leads to changes in network connectivity over time (Krystal & Anticevic, 2015). It is counterintuitive to believe that constitutive, prolonged NMDAR hypofunction, resulting in excessive glutamate release in key circuits of the brain early in life and through adolescence, would result in a static cellular and behavioral phenotype during all stages of adulthood. Put another way, it is likely that prolonged increases in cellular excitability leads to progressive changes in circuitry, resulting in changes in behavior over time. In support of this, there are reports indicating a progressive loss of dendritic spine density in NR1 KD mice compared to WT littermate controls (Ramsey et al., 2011), and though unpublished, our group has observed progressive changes in locomotor activity and pyramidal cell firing rate that coincide with the age of the animal. Additionally, recent clinical studies by (Anticevic et al., 2015) have demonstrated that acute treatment with the NMDAR antagonist ketamine in healthy humans can induce a state of mPFC disinhibition, or hyperconnectivity, that is also observed in early course schizophrenic patients. Moreover, this hyperconnectivity observed in early course patients dissipates over time, and is not observed in patients that have chronic schizophrenia. With this in mind, it is important to entertain the idea that schizophrenia may be a progressive disease with an ever-evolving phenotype, and the animal models chosen may imitate different stages of the disease.

If schizophrenia is, in fact, a progressive disease, it is crucial that we make strides towards understanding the changes in circuitry over time to better inform the type of therapy needed. For example, administration of an M_4 PAM in early stages of the disease may produce an antipsychotic-like phenotype and elicit some degree of cognitive enhancement through modulation of PFC circuitry. Alternatively, a patient that has chronic schizophrenia with a reduction in glutamatergic signaling in the PFC may experience adverse consequences if an M_4 PAM was administered. Of course, this is an over simplified scenario, and M_4 PAM treatment could be efficacious in both through a different mechanism, such as modulating sleep/wake architecture. Regardless, treating schizophrenia as a progressive disease should alter the design of clinical trials for novel antipsychotics in favor of patient stratification. This should decrease the rate of clinical candidate failure in human trials, and should better serve patients with schizophrenia by more effectively addressing the symptoms that are present at their stage of the disease.

Perhaps most excitingly, this model would suggest that early intervention with a targeted pharmacotherapeutic that reduces cortical excitation, such as an M_4 PAM, could prevent some of the neuroplastic changes that are thought to occur as a result of the disease. In this sense, early treatment with an M_4 PAM could actually be disease-altering and neuroprotective. One mechanism for this preventative action could be through direct modulation of glutamate levels in the PFC, in much the same way $mGlu_{2/3}$ activators or 5-HT_{2A} antagonists elicit this response (Marek et al., 2000; Moghaddam & Adams, 1998). It will be important to perform preclinical studies in animal models of hyperfrontality or disinhibition to test this hypothesis. For example, based on the work described above, repeated administration of an M_4 PAM should be tolerated well and allow for chronic

dosing to occur in NR1 KD mice. As stated previously, these mice undergo a progressive loss in dendritic spine density, resulting in altered glutamatergic function that is consistent with clinical literature (Garey et al., 1998; Sweet et al., 2009). Since we have shown that acute administration of an M_4 PAM results in altered glutamatergic signaling in these animals, it is fair to postulate that chronic treatment could prevent spine loss if the decrease in density was associated with hyperglutamatergic signaling. Studies like these will allow for the investigation of the effects of M_4 PAMs in models of early-course schizophrenia and may predict the disease-altering properties of muscarinic ligands and other novel mechanisms that modulate cortical signaling.

In sum, we have demonstrated both the antipsychotic-like effects and cognitive enhancing properties of M_1 and M_4 PAMs, through modulation of dopaminergic or glutamatergic circuitry, or both. Additionally, we have shown efficacy in a broad range of assays utilizing pharmacological and genetic approaches to model the complex symptoms of schizophrenia. Taken together, the findings reported in this dissertation provide strong support for the development of selective M_1 and M_4 PAMs for the treatment of schizophrenia.

APPENDIX A

EFFECTS OF VU0467154 ON AMPHETAMINE- OR MK-801-INDUCED DEFICITS IN 5CSRT TASK

Methods

Five Choice Serial Reaction Time (5CSRT) task.

This method is based on previous studies by (Amitai & Markou, 2009; Shannon et al., 2007) using a within subject design in male adult Sprague-Dawley rats. Rats were food-restricted to approximately 85% of their free-feeding weights by food presented during experimental sessions and supplemental feeding after the experimental sessions. The apparatus consisted of operant conditioning chambers located within sound and light-attenuating enclosures (model ENV-009, MED Associates Inc., St Albans, Vermont, USA). Each chamber was equipped with a white house light centered near the top of the front panel and a tone generator near the top on the right-hand side of the front panel. Five nosepoke apertures were located 2.5 cm above the chamber floor with 1.5 cm between nosepoke aperture with a 28 V direct current and 100 mA stimulus light within each nosepoke apertures. A pellet dispenser was centered on the opposite wall and delivered 45-mg dustless precision pellets (Bioserv, Frenchtown, New Jersey, USA). Operation of the house and stimulus lights, pellet dispenser, tone generator, and recording of data were controlled by a computer using Med-State Notation software (version 2; MED Associates). Rats were trained to discriminate a brief visual stimulus presented randomly in one of the five nosepoke apertures. Under the final schedule contingencies, each session began with a 1-min acclimatization period during which the chamber was dark and responding had no scheduled consequences. The beginning of each trial was

signaled by the illumination of the house light. A response on any nosepoke aperture after the onset of the house light, but before the presentation of a stimulus light immediately terminated the house light and initiated a 5-s intertrial interval (ITI) during which the chamber was dark and responding had no scheduled consequences. Responses during the prestimulus interval were termed anticipatory responses. In the absence of an anticipatory response, after an average of 6.5 s (5.0–8.0 s) following the onset of the house light, a stimulus light was illuminated above one of the five nosepoke apertures in random order. During training sessions, the stimulus light remained illuminated for a maximum of 2 s. During test sessions, the stimulus light remained illuminated for a maximum of either 0.2, 1.0, or 2.0 s, in randomized order and the two durations were presented an equal number of times. A response on the nosepoke aperture below which the stimulus is presented within 5 s of the onset of the stimulus ('response window') immediately terminated the trial, and resulted in the presentation of a food pellet, 0.2 s presentation of a tone, and initiated a 5-s ITI. An incorrect response during the response window immediately terminated the trial and initiated a 5-s ITI. Responses during the ITI were counted but have no scheduled consequences; the next trial began immediately after the end of the 5-s ITI. Sessions were conducted 4 days per week and each session duration was 30 min or until 100 trials had been completed, whichever occurred first each session. Data was analyzed for the following parameters: 1) Accuracy: the number of correct responses divided by the sum of correct and incorrect responses([# correct responses/(# correct responses + # incorrect responses)] × 100); 2) Percent Correct Responses (total # correct responses/total # of trials) × 100); 3) Percent Incorrect Responses (total # incorrect responses/total # of trials) × 100); 4) Percent Omissions (total # omissions/total # of

trials) × 100); 5) Premature or anticipatory responses (total # of responses performed during the ITI, before presentation of the light stimulus); 6) perseverative responses, further responses into the apertures after the performance of a correct response, but before reward retrieval from the magazine/initiation of a new trial resulting in a timeout and no food reward. Data was analyzed using a one-way analysis of variance and if significant ($p<0.05$), all dose groups were compared to the vehicle group using a Dunnett's test. A difference will be considered significant when $p \leq 0.05$.

Results

Rats were trained to baseline performance values over the course of ~30 days, which was considered 2 consecutive days above 70% correct when tested at a 2 s stimulus interval. All studies were conducted using a within subject design. On testing day, rats received one of 3 dosing schedules: vehicle/vehicle (10% tween 80, i.p., 60 min pretreatment/sterile water, s.c., 30 min pretreatment), vehicle/AMPH (10% tween 80, i.p., 60 min pretreatment/0.732 mg/kg AMPH, s.c., 30 min pretreatment), or VU0467154/AMPH (1, 3, or 5.6 mg/kg, i.p., 60 min pretreatment/0.732 mg/kg AMPH, s.c., 30 min pretreatment). In the same cohort of animals, following a significant washout period, rats received one of 3 dosing schedules: vehicle/vehicle (10% tween 80, i.p., 60 min pretreatment/sterile water, s.c., 30 min pretreatment), vehicle/MK-801 (10% tween 80, i.p., 60 min pretreatment/0.1 mg/kg MK-801, s.c., 30 min pretreatment), or VU0467154/MK-801 (0.56, 1, 3, or 5.6 mg/kg, i.p., 60 min pretreatment/0.1 mg/kg MK-801, s.c., 30 min pretreatment). Vehicle treated animals responded at levels consistent with 2 s stimulus presentation on training days, with testing responses at ~70% (Figure

38). When the stimulus presentation interval was decreased, however, there was a significant decrease in % correct responding and an increase in % omissions. AMPH administration alone resulted in a significant decrease in % correct responding across all stimulus presentation times (Figures 38-40). Pretreatment with VU0467154 was able to attenuate the response to AMPH and MK-801 responses at select doses and stimulus presentation intervals. While these data are promising, the lack of consistency across dose and presentation interval makes interpreting the data difficult. Potential problems with the study design may include the dose of psychostimulant used to induce disruptions in performance, pretreatment times with VU0467154, or the advanced age of the rats (> 1 year old). Also worth noting, these rats had a complex drug history including previous dosing with a range of psychostimulants, muscarinic agonists and modulators, and metabotropic glutamate receptor ligands.

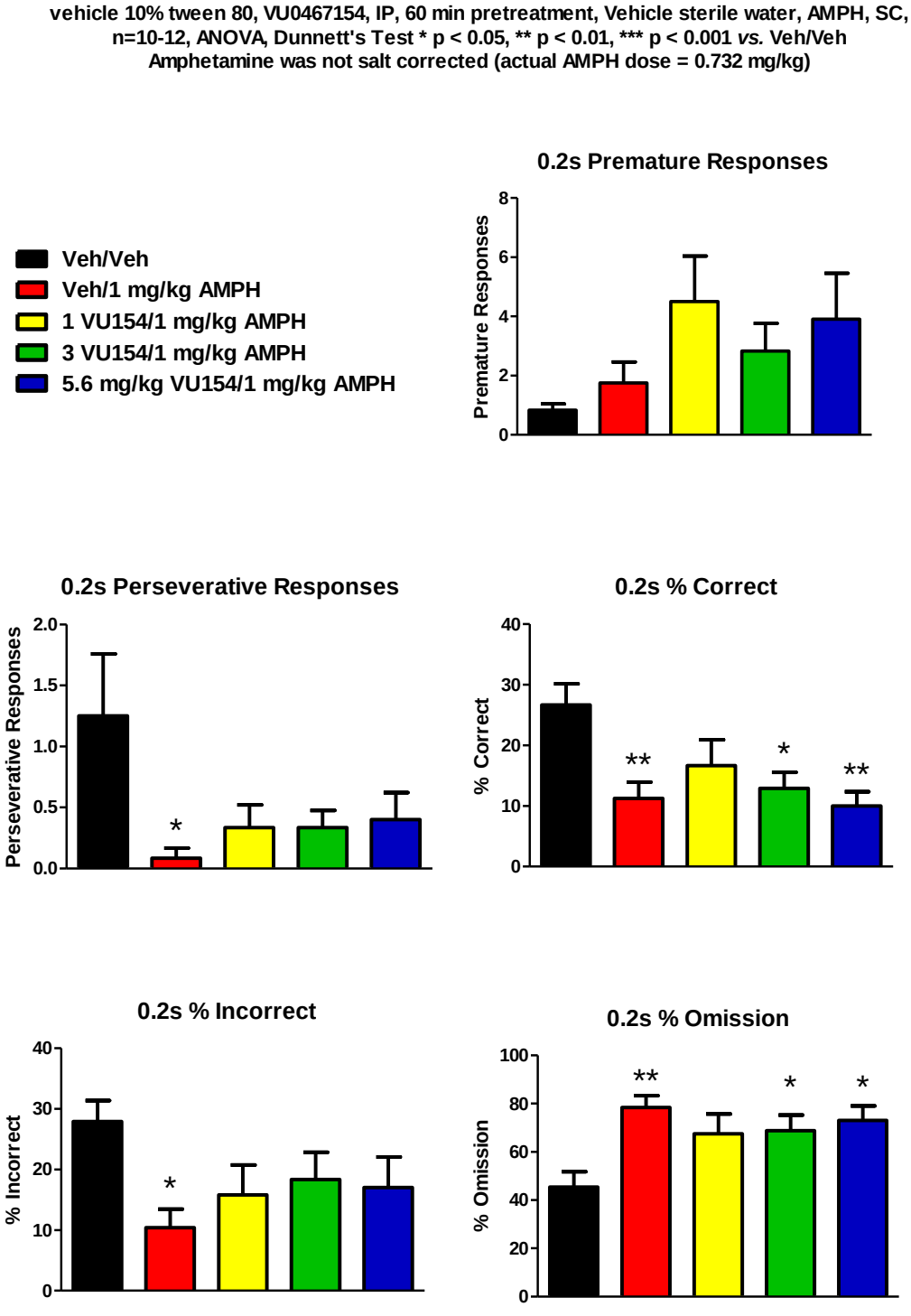


Figure 38. Effects of an M_4 PAM on amphetamine-induced disruptions in 5CSRT task performance at 0.2 s stimulus presentation interval.

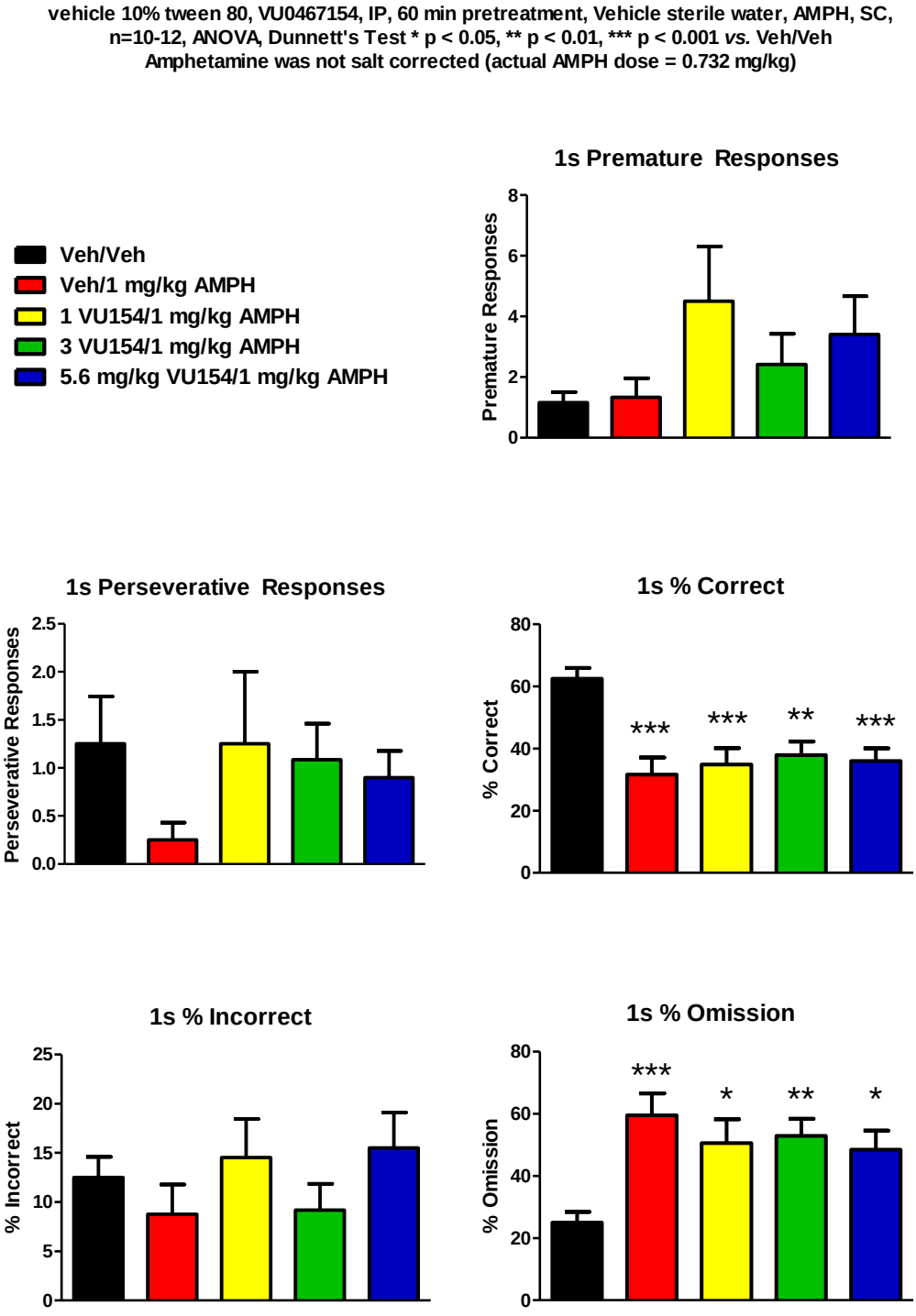


Figure 39. Effects of an M_4 PAM on amphetamine-induced disruptions in 5CSRT task performance at 1.0 s stimulus presentation interval.

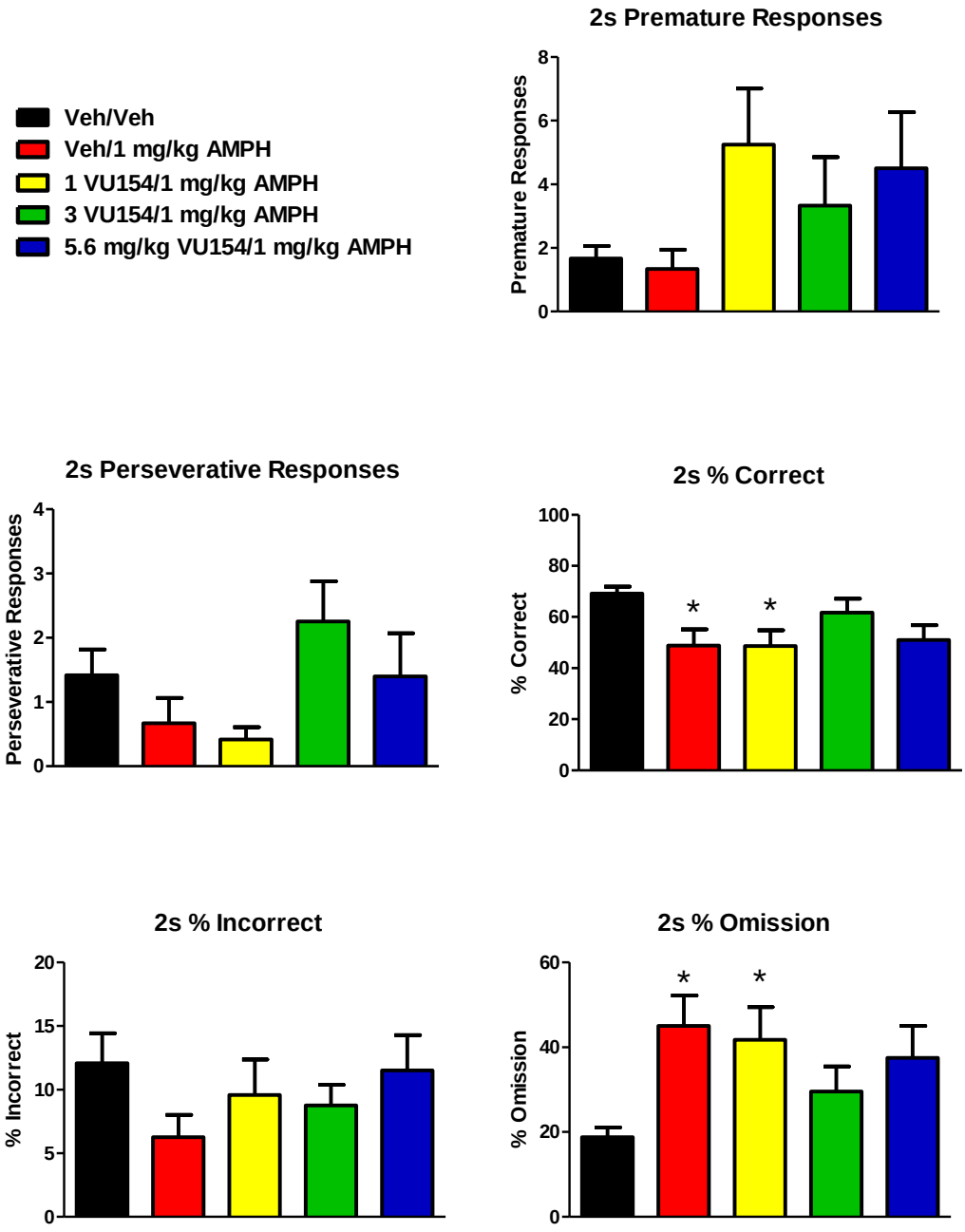


Figure 40. Effects of an M_4 PAM on amphetamine-induced disruptions in 5CSRT task performance at 2.0 s stimulus presentation interval.

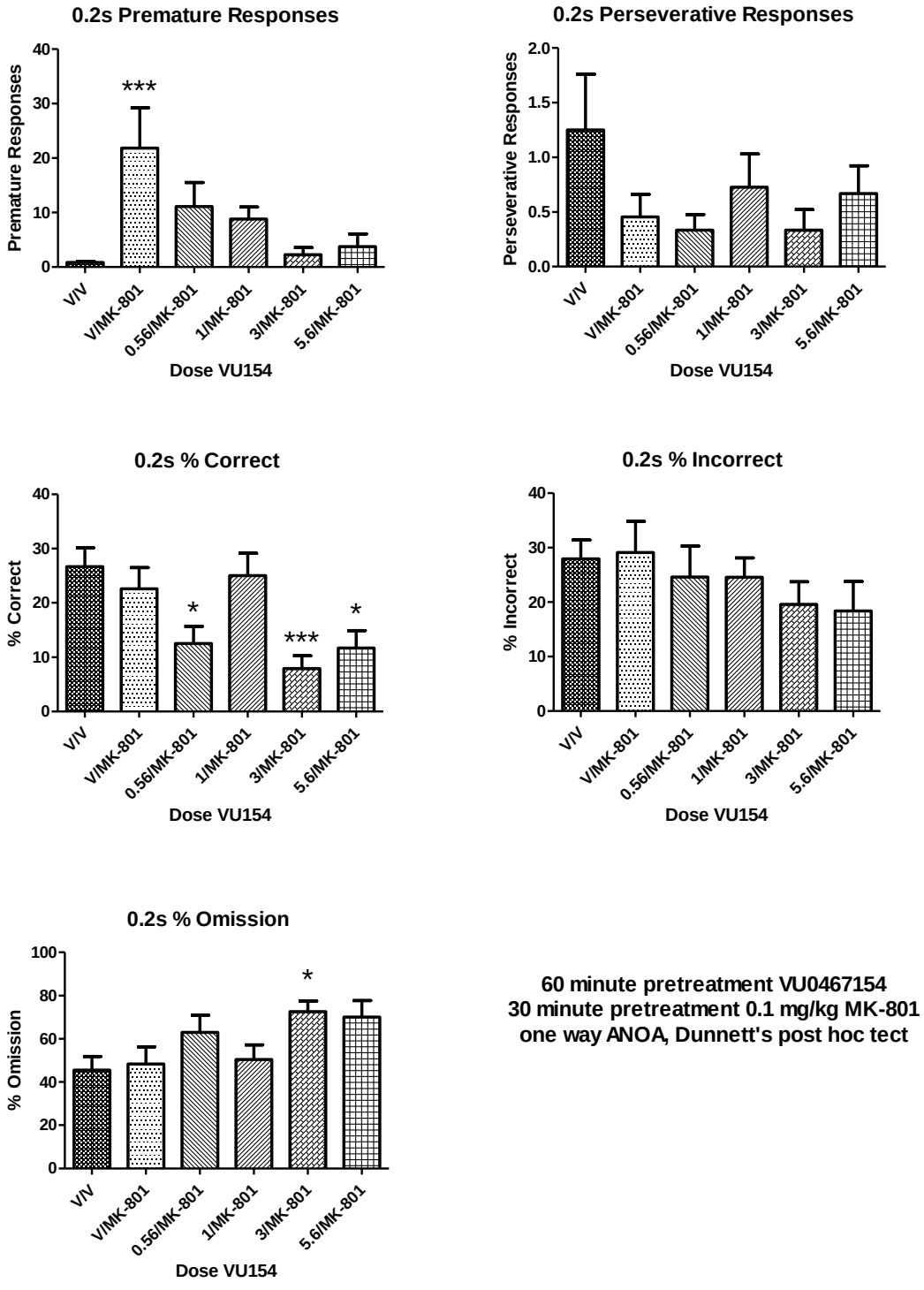


Figure 41. Effects of an M_4 PAM on MK-801-induced disruptions in 5CSRT task performance at 0.2 s stimulus presentation interval.

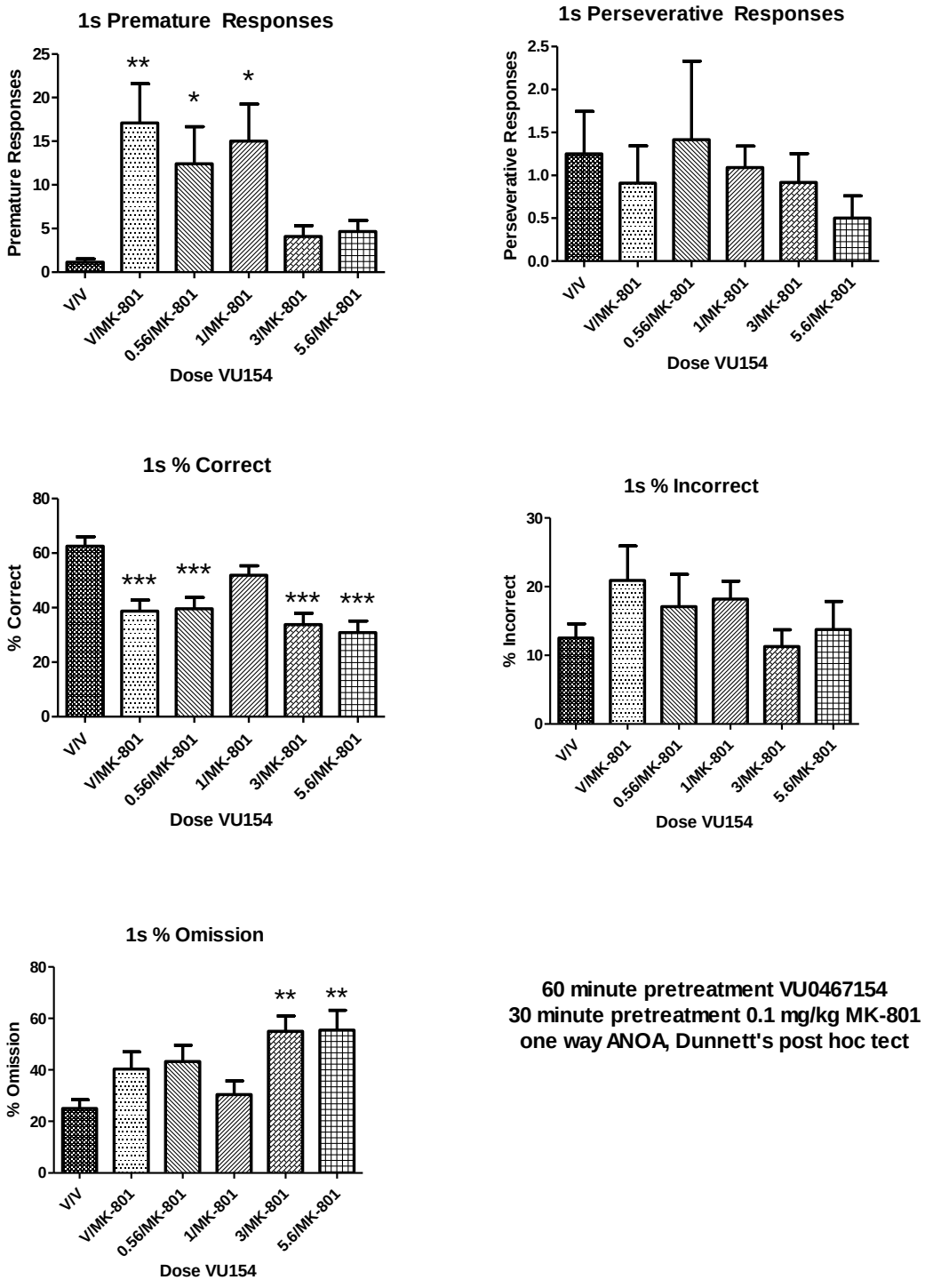


Figure 42. Effects of an M_4 PAM on MK-801-induced disruptions in 5CSRT task performance at 1.0 s stimulus presentation interval.

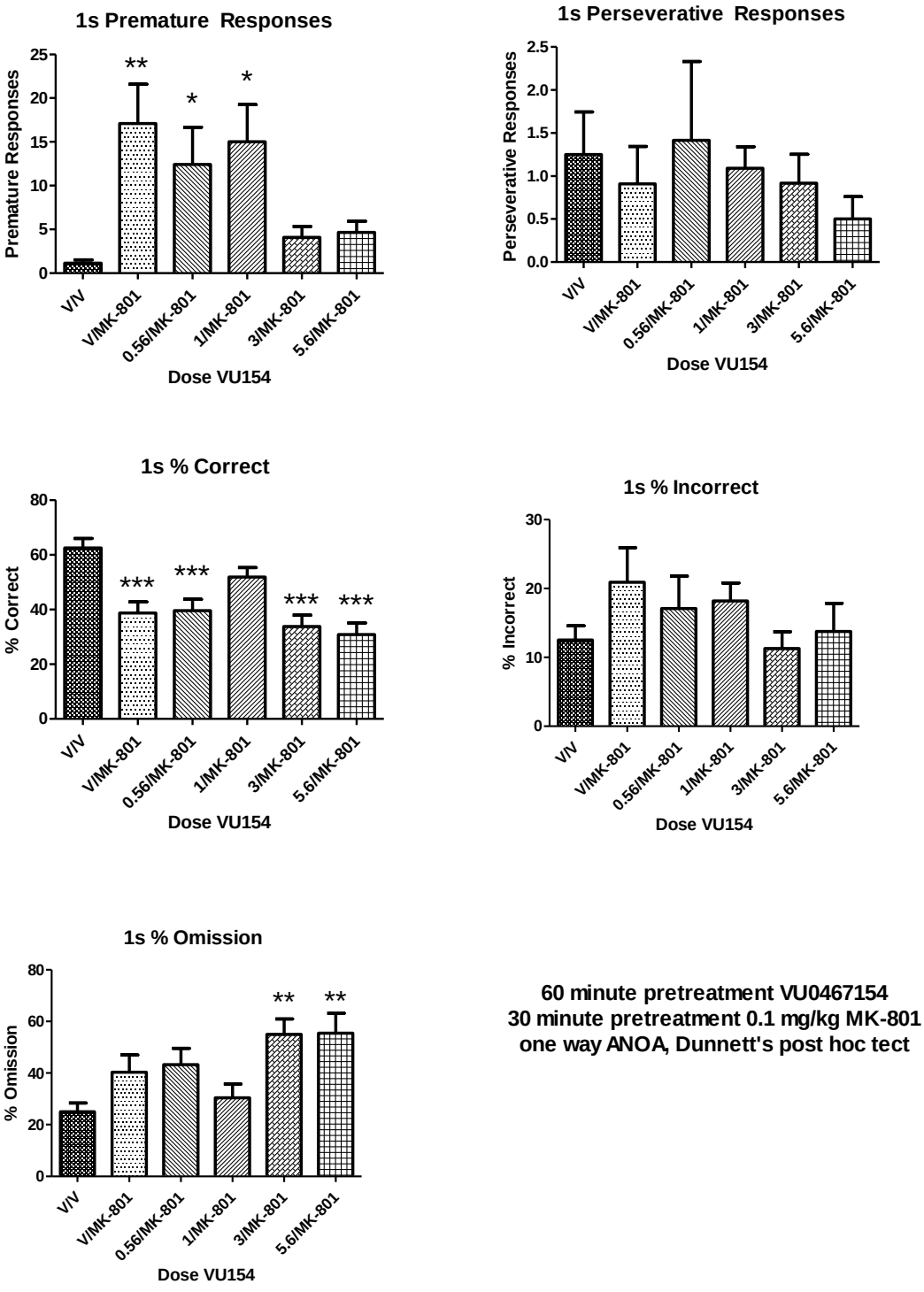


Figure 43. Effects of an M_4 PAM on MK-801-induced disruptions in 5CSRT task performance at 2.0 s stimulus presentation interval.

APPENDIX B

EFFECTS OF VU0467154 ON MK-801-INDUCED HYPERLOCOMOTOR ACTIVITY WITH ALTERED DOSING SCHEDULE

Methods

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 (Jones et al., 2008). To determine the effects of VU0467154 on reversing MK-801-induced hyperlocomotion, rats were habituated in the open field for 30 min, followed by administration of vehicle (0.9% saline) or MK-801 (0.18 mg/kg, IP). After an additional 30 min, (10% Tween 80 in sterile water) or VU0467154 (30 mg/kg, IP) was injected, and locomotor activity was recorded for another 120 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. Ttime course data (means ± SEM) was analyzed by two-way ANOVA and *post hoc* comparisons were made by Dunnett's test using GraphPad Prism.

Results

This study was performed to assess the ability of an M_4 PAM to reverse MK801-induced hyperlocomotor activity when administered following the onset of MK-801 response. Traditionally, our group, and others, pretreats rodents with a compound that has potential antipsychotic-like activity based on the pharmacokinetics of the compound. The blockade of psychostimulant-induced hyperlocomotor response is then calculated

based on a vehicle treated control. In this experiment, rats were administered MK-801 (0.18 mg/kg) 30 minutes prior to VU0467154, to measure the ability of the compound to reverse the locomotor response induced by MK-801. Consistent with previously reports, MK-801 induced a robust increase in locomotor activity within 15 mins of administration (Figure 44). However, unlike previously published pretreatment paradigms, VU0467154 administration following the onset of MK-801 response was not able to significantly reduce locomotor activity. This study highlights the necessity to dose a potential antipsychotic-like compound in accordance with the known pharmacokinetics properties of the compound.

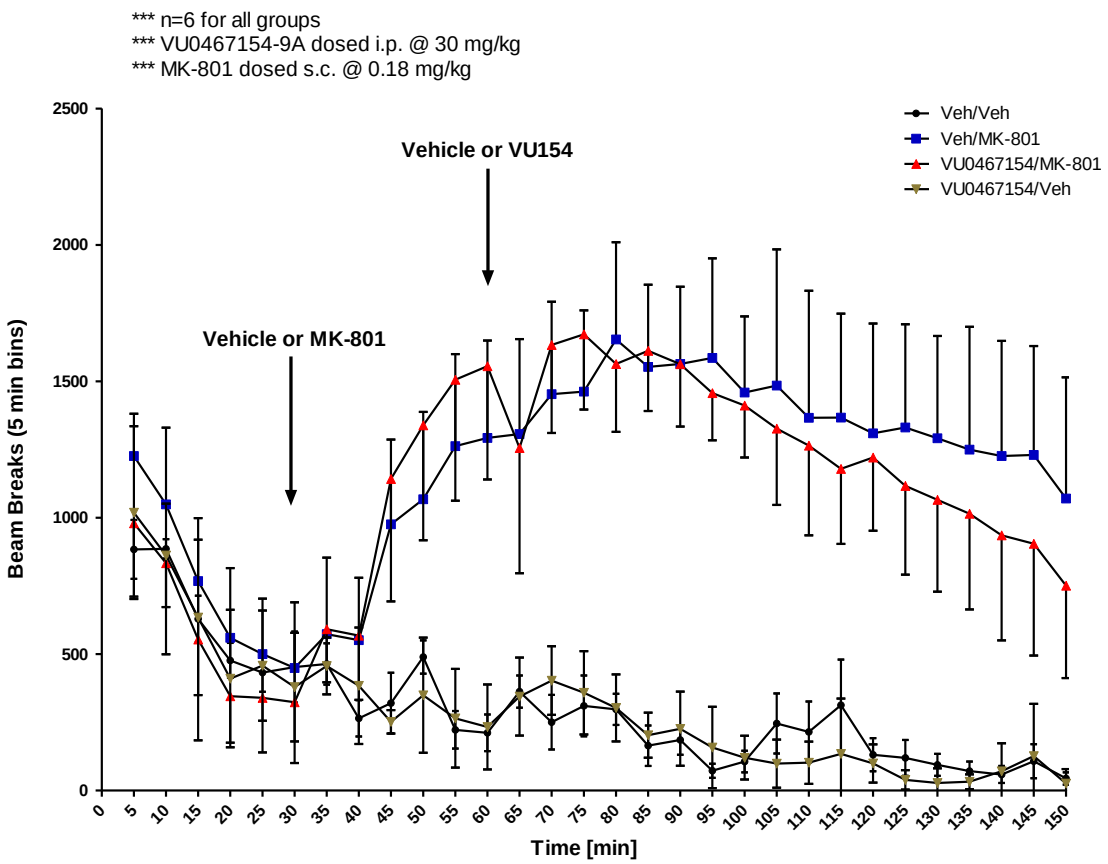


Figure 44. Effects of an M_4 PAM on MK-801-induced hyperlocomotion when administered 30 minutes following Mk-801. MK-801 induced a robust increase in locomotor activity within 15 mins of administration. However, unlike previously published pretreatment paradigms, VU0467154 administration following the onset of MK-801 response was not able to significantly reduce locomotor activity (ANOVA followed by Bonferroni's post hoc test).

APPENDIX C

IN VIVO MICRODIALYSIS IN WT AND NR1 KD MICE

Methods

Mice (n = 8-9 per genotype) were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). A guide cannula (CMA7, Harvard Apparatus, USA) was placed above the medial prefrontal cortex (+2.0 AP, ± 0.7 ML from Bregma and -1.0 DV from dura) and secured to the skull with glass ionomer cement (Instech Laboratories). Following a 5-7 day recovery period, animals were placed in individual activity chambers and a microdialysis probe (CMA7 microdialysis, USA) with an active length of 2 mm was inserted into the guide cannula. The probe was then perfused at a rate of 1.0 μL/min with artificial cerebral spinal fluid (149 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, pH 7.2. After a 2-hour equilibration period dialysate samples were collected every ten minutes (10 μL fractions) for a total of 4 hours. **Benzoylation Reaction**. Calibration standard or dialysate samples (5 μL) were added to 2.5 μL of 100 mM sodium borate buffer 2.5 μL of benzoyl chloride (2% in acetonitrile). The mixture was vortexed, and 2.5 μL of internal standard was added before LC-MS analysis. **HPLC-MS Analysis.** Dialysate sample components, including glutamate and acetylchoine, were separated on a Waters BEH C18 column (1 mm × 100 mm, 1.7 μm, 13 nm pore size, maintained at using 27 °C, using.a Waters nanoAcquity HPLC system (Milford, MA). Following sample injection (9 μL), analytes were separated at a flow rate of 100 μL/min, by gradient elution (mobile phase A: 10 mM ammonium

formate and 0.15% (v/v) formic acid in water; mobile phase B [acetonitrile]. Analytes were then quantitated using a Waters/Micromass Quattro Ultima triple quadrupole mass spectrometer (for a more detailed description see (Song et al., 2012)). Basal levels of extracellular glutamate and acetylcholine were compared between genotypes using a student's t-test.

Results

Basal levels of extracellular glutamate and acetylcholine in WT and NR1 KD mice

Preliminary *in vivo* microdialysis analysis of extracellular glutamate and acetylcholine levels were measured in mPFC of NR1 KD and WT mice. NR1 KD mice appear to have increased basal glutamate and acetylcholine levels compared to littermate controls, though there is a large amount of variability between animals that prevents statistical significance at this point. This is likely due to differences in expression levels of the NR1 subunit in the knockdown animals. This variability has been documented by our group, and others, and is most obvious when monitoring behavioral responses, such as basal locomotor activity or performance in CF. There is also potential for variability in the derivatization of microdialysis samples prior to analysis using HPLC/MS. Finally, the use of an antioxidant mixture containing a cholinesterase inhibitor in the collection wells would aid in the stability of the sample and allow for a greater degree of consistency between samples.

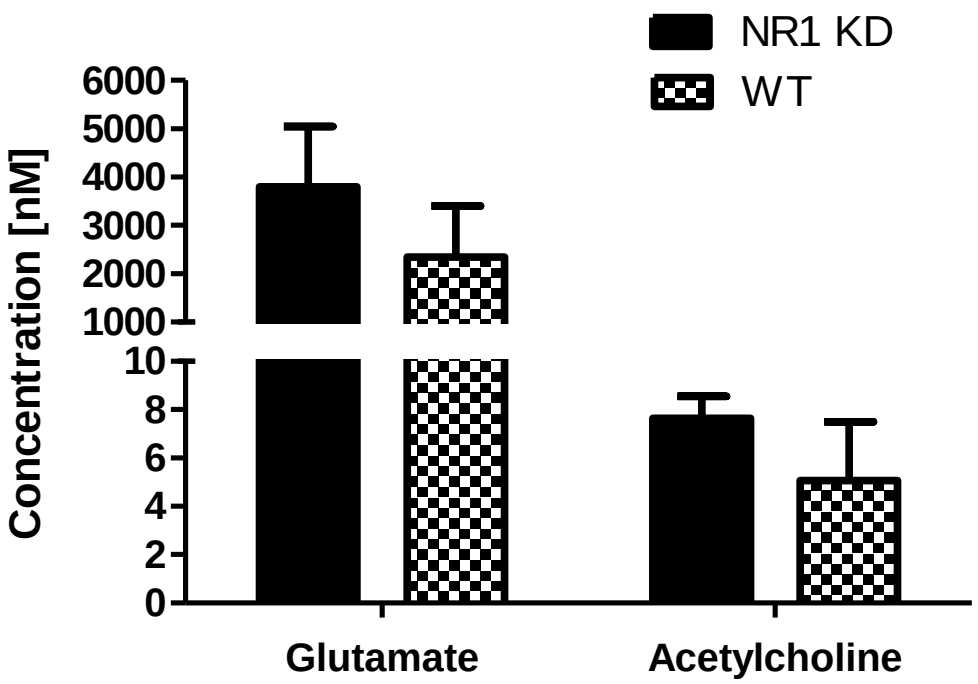


Figure 45. Preliminary *in vivo* microdialysis analysis of extracellular glutamate and acetylcholine levels in mPFC of NR1 KD and WT mice. NR1 KD mice have a trend towards increased basal glutamate and acetylcholine levels compared to littermate controls. n = 8-9 animals, student's t-test, n.s.).

APPENDIX D

PROGRESSIVE RATIO STUDIES IN WILDTYPE AND M_4 KO MICE

Methods

To assess the reinforcing strength of the liquid reinforcer between wild-type and M_4 KO mice, we trained a separate cohort of wild-type and M_4 KO mice to respond on a progressive ratio schedule. Mice (age 9-12 weeks at training; n=11-12/genotype) were maintained at 85% their free-feeding weight and first trained to respond via a nosepoke in operant chambers (Med Associates) with 3 nosepoke holes on one wall and a reward receptacle on the opposite wall to allow reinforcement delivery from a dipper. Mice were initially trained such that a single response in the middle nosepoke hole when a light was illuminated would be reinforced via delivery of 0.2 mls of 33% diluted Ensure (fixed ratio 1 schedule of reinforcement). The dipper would remain elevated until the mouse entered the reward receptacle and for 5 seconds thereafter. Sessions lasted 1 hour or until completion of 100 trials. The fixed ratio (FR) was increased to 10 responses over the course of subsequent sessions. When mice completed greater than 50 trials under a FR10 schedule for a minimum of three days, the reinforcement schedule was switched to a progressive ratio. The number of responses necessary for reinforcement delivery increased following each completed ratio based on the equation described by (Richardson & Roberts, 1996) ratio= [5e^injection number x 0.2)]-5. The first 10 ratios in the series were 1, 2, 4, 6, 9, 12, 15, 20, 25, 32. Following each completed ratio, the stimulus light was extinguished for 5 sec (ITI). Sessions lasted for 2 hours or until a 20-min period elapsed during which a ratio was not completed; the last ratio completed was termed the break point and served as the dependent measure to assess reinforcing strength between

genotypes. Responding was initially maintained by 33% diluted Ensure and then a concentration-response curve was determined (water, 10%, 33% 100% Ensure) in random order. Each concentration was available for a minimum of 5 days and a 3-day stable average was determined such that the number of reinforcers delivered did not deviate from the mean by more than 2. If stability was not achieved within 10 sessions, a 5-day average was calculated. Following each determination, the reinforcer concentration was returned to 33% for 2-3 days to ensure that baseline responding was similar prior to a new concentration determination. The primary dependent variable was the number of ratios completed at each dose.

Results

A two-way non-repeated measures ANOVA examined effects of concentration and genotype followed by Bonferonni post-hoc comparisons, $p<0.05$. There was a main effect of genotype ($F_{1,84}=24.49$, $p<0.0001$) and dose ($F_{3,84}=77.84$, $p<0.0001$) and a significant interaction ($F_{3,84}=3.74$, $p<0.05$) (Figure 46). Number of reinforcers and completed ratios increased in a concentration-dependent manner in both genotypes. M_4 KO mice received a higher number of reinforcers than wild-type mice under vehicle (water; $p<0.01$), 10 ($p<0.01$) and 33 % ($p<0.05$) Ensure.

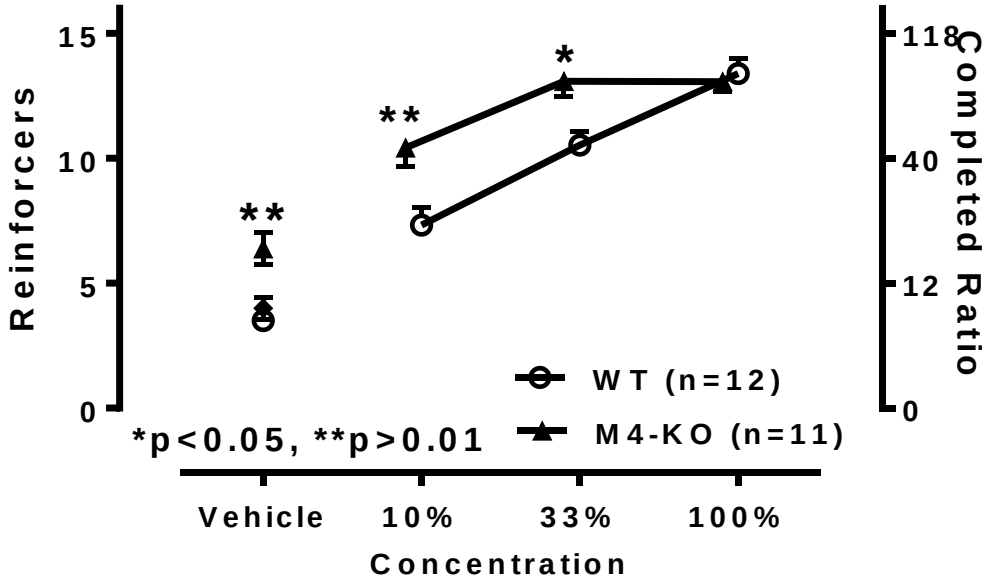


Figure 46. Assessing relative reinforcing strength of the liquid reinforcer in wild-type and M_4 KO mice under progressive ratio schedule of reinforcement.

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