Published in final edited form as:

Neuropharmacology. 2016 March ; 102: 244–253. doi:10.1016/j.neuropharm.2015.11.016.

State-Dependent Alterations in Sleep/wake Architecture Elicited by the M₄ PAM VU0467154- Relation to Antipsychotic-like Drug Effects

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Abstract

Accumulating evidence indicates direct relationships between sleep abnormalities and the severity and prevalence of other symptom clusters in schizophrenia. Assessment of potential state-dependent alterations in sleep architecture and arousal relative to antipsychotic-like activity is critical for the development of novel antipsychotic drugs (APDs). Recently, we reported that VU0467154, a selective positive allosteric modulator (PAM) of the M₄ muscarinic acetylcholine receptor (mAChR), exhibits robust APD-like and cognitive enhancing activity in rodents. However, the state-dependent effects of VU0467154 on sleep architecture and arousal have not been examined. Using polysomnography and quantitative electroencephalographic recordings from subcranial electrodes in rats, we evaluated the effects of VU0467154, in comparison with the

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Financial Disclosures: The authors declare the following competing financial interest(s): Over the past two years, C.W.L. consulted for Abbott. M.B., T.M.B., M.J.N., C.M.N., J.S.D., M.R.W., C.W.L., P.J.C., and C.K.J. received research/salary support from AstraZeneca and/or Bristol Myers Squibb. T.M.B., C.M.N., J.S.D., M.R.W., C.W.L., and P.J.C. are inventors on multiple composition of matter patents protecting allosteric modulators of GPCRs. M.E.D., J.D., N.J.B., and M.W.W. are employees of AstraZeneca. M.I. is an employee of Proteostasis Therapeutics and was formerly employed by AstraZeneca. The remaining authors declare no competing financial interests.

Supplementary information is available at the *Neuropharmacology* website.

A portion of these studies was presented at the 2013 and 2014 Experimental Biology, 2014 Winter Brain Research Conference, and 2014 American College of Neuropsychopharmacology annual meetings.

atypical APD clozapine and the M₁/M₄-preferring mAChR agonist xanomeline. VU0467154 induced state-dependent alterations in sleep architecture and arousal by delaying Rapid Eye Movement (REM) sleep onset, selectively increased cumulative duration of total and Non-Rapid Eye Movement (NREM) sleep, and increased arousal during waking periods. Clozapine decreased arousal during wake, increased cumulative NREM, and decreased REM sleep. In contrast, xanomeline increased time awake and arousal during wake, but reduced slow wave activity during NREM sleep. Additionally, in combination with the *N*-methyl-D-aspartate subtype of glutamate receptor (NMDAR) antagonist MK-801, modeling NMDAR hypofunction thought to underlie many symptoms in schizophrenia, both VU0467154 and clozapine attenuated MK-801-induced elevations in high frequency gamma power consistent with an APD-like mechanism of action. These findings suggest that selective M₄ PAMs may represent a novel mechanism for treating multiple symptoms of schizophrenia, including disruptions in sleep architecture without a sedative profile.

Keywords

VU0467154; M₄ muscarinic acetylcholine receptor; positive allosteric modulator; electroencephalography; clozapine; xanomeline

1. Introduction

Abnormal sleep architecture, including insomnia, reduced quality or duration of slow wave sleep (SWS) and increased Rapid Eye Movement (REM) sleep, are commonly reported in both medicated and un-medicated patients with schizophrenia and linked with the prevalence and severity of the other symptom clusters (for reviews see Cohrs, 2008; Sprecher et al., 2015). Sleep disturbances and decreased REM latency often precede and are present during acute psychosis (Pritchett et al., 2012; Sprecher et al., 2015), while decreases in SWS duration or quality are associated with the negative and cognitive symptoms of the disorder (2006; Goder et al., 2004; Keshavan et al., 1995b; Yang and Winkelman, 2006). Recent studies suggest that normalizing sleep disturbances associated with schizophrenia may also improve other symptom clusters (Manoach and Stickgold, 2009). For example, declarative memory, one of the most disrupted cognitive domain in schizophrenia (Green et al., 2000) is improved following SWS-rich sleep compared to REM-rich sleep in healthy subjects (Plihal and Born, 1997). Moreover, declarative memory is improved by increasing slow wave activity (SWA), a neurophysiological correlate of sleep quality (Cohrs, 2008; Steiger and Kimura, 2010) during SWS via transcranial direct current stimulation in patients with schizophrenia (Goder et al., 2013). Cognitive performance is a critical predictor of overall functional outcome (Bobes et al., 2007; Green, 1996; Green et al., 2004). Thus, strategies to improve cognitive performance, including modifying abnormal sleep architecture, may represent a novel treatment approach for schizophrenia.

In addition to polysomnography, quantitative electroencephalography (qEEG) approaches have correlated aberrant brain oscillation patterns with specific symptoms in unmedicated schizophrenia patients and adverse side effect profiles of antipsychotic drugs (APDs) in medicated patients (Boutros et al., 2008; 2014; Goder et al., 2006; Keshavan et al., 1995a;

Wichniak et al., 2006). For example, elevated power in the high frequency gamma band (e.g., >30 Hz in humans) is associated with positive symptoms, whereas lower gamma power at rest and reduced elevations in gamma power during cognitive testing is linked to cognitive impairments (Baldeweg et al., 1998; Chen et al., 2014; Lee et al., 2003; Uhlhaas and Singer, 2014). To date, clinically available APDs provide modest to no therapeutic benefit for many of the symptoms observed in schizophrenia patients, including negative symptoms and cognitive deficits (Krystal et al., 2008; Reichenberg and Harvey, 2007). At therapeutically relevant doses, many APDs, including the atypical APD clozapine, increase total sleep time and SWA. However, these potentially beneficial effects during sleep persist during wake as a general EEG slowing (e.g. shift in power from high to low frequencies) that correlate with problematic sedating effects in healthy subjects and schizophrenia patients (Freudenreich et al., 1997; Roubicek and Major, 1977; Yoshimura et al., 2007) that may contribute to cognitive impairments. These studies highlight the importance of understanding state-dependent alterations in sleep architecture and arousal relative to APD-like and cognition enhancing activity in the development of novel APDs.

One promising approach for the development of novel APDs involves modulation of the muscarinic cholinergic system (Jones et al., 2012), which is regulated by five different subtypes of muscarinic acetylcholine receptors (mAChRs), termed M₁-M₅ (Bonner et al., 1987; 1988) and plays a critical role in regulating arousal, mood, cognition, and sleep/wake architecture (Graef et al., 2011; Platt and Riedel, 2011). Nonselective mAChR agonists and acetylcholinesterase inhibitors increase arousal, cognition, and REM bouts and/or duration, but reduce NREM sleep duration (Nissen et al., 2006; Riemann et al., 1994). In clinical studies, the M₁/M₄-preferring mAChR agonist xanomeline reduced the behavioral disturbances and psychotic symptoms observed in Alzheimer's disease and schizophrenia patients, respectively (Bodick et al., 1997a; 1997b; Shekhar et al., 2008). However, xanomeline and 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). Over the last decade, we and others have developed a novel strategy for activation of individual mAChR subtypes, particularly the M₄ subtype, using highly selective positive allosteric modulators (PAMs) (Brady et al., 2008; Bubser et al., 2014; Byun et al., 2014). These M₄ PAMs do not activate the M₄ mAChR directly, but potentiate the response of the receptor to ACh, thereby enhancing activity-dependent signaling through allosteric binding sites that are more topographically distinct and less highly-conserved than the orthosteric ACh binding site (Conn et al., 2009). Selective M₄ PAMs produce the APD-like profile previously reported with xanomeline (Brady et al., 2008; Bubser et al., 2014; Byun et al., 2014; Mirza et al., 2003). In addition, the M₄ PAM VU0467154 produces cognitive-enhancing effects when administered alone on measures of learning and memory and reverses MK-801-induced hyperlocomotion and cognitive disruptions (Bubser et al., 2014). MK-801, an antagonist of the *N*-methyl-D-aspartate subtype of the glutamate receptor (NMDAR), is often used to model neurophysiological, neurochemical, and behavioral disruptions associated with NMDAR hypofunction, which are thought to underlie many of the symptoms in schizophrenia (Anticevic et al., 2015; Blot et al., 2013; Coyle et al., 2012). The above studies suggest that M₄ PAMs may provide efficacy for multiple symptom clusters associated with schizophrenia. However, the effects of selective M₄ PAMs on sleep

architecture and arousal relative to antipsychotic-like and cognitive enhancing activity remain unknown.

In the present studies, we examined the effects of the M₄ PAM VU0467154, in comparison with clozapine and xanomeline, on sleep architecture and arousal using EEG in freely moving rats. Specifically, we confined our analyses to examine aspects of sleep/wake architecture known to be disrupted in patients with schizophrenia and/or are directly affected by APDs. Compounds were evaluated during the light period to examine wake-promoting or arousal enhancing effects and to examine changes in duration of REM/non-REM sleep and SWS quality. Compounds were evaluated during the dark period to examine sleep-promoting or sedative effects. We also extended previous studies to examine the ability of these compounds to attenuate MK-801-induced elevations in high frequency gamma power (50-100 Hz), a putative biomarker of NMDAR hypofunction and potential antipsychotic-like activity. Previous studies have demonstrated that NMDAR antagonists induce cognitive impairments and increase high frequency gamma power in healthy humans and exacerbate cognitive impairments in patients with schizophrenia (Coyle et al., 2012; Hong et al., 2010; Kocsis et al., 2013; Tsai and Coyle, 2002). VU0467154 produced selective increases in the duration, without disrupting quality, of NREM sleep and, in contrast to clozapine, increased arousal during wake. VU0467154 also reversed MK-801-induced elevations in gamma power consistent with APD-like activity (Hiyoshi et al., 2014). The state-dependent actions of the M₄ PAM VU0467154 represent a potentially unique therapeutic profile for treating many of the symptoms in schizophrenia patients, including the abnormalities in sleep architecture and arousal, without the sedation observed with other APDs.

2. Methods and Materials

2.1 Subjects

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) maintained on a 12-h light:12-h dark cycle with *ad libitum* access to food and water served as subjects. All 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*.

2.2. Surgery

Rats (250-275 g) were surgically implanted under isoflurane anesthesia with a telemetric transmitter (4-ET, Data Sciences International [DSI], Minneapolis, MN) for recording EEG, electromyographic (EMG), and motor activity as previously described (Nedelcovych et al., 2015). Briefly, three sets of leads were placed bilaterally in contact with the dura to record from cortical regions corresponding with the frontal, parietal and occipital cortices (+3 mm, -3 mm and -6 mm from Bregma, respectively and ± 2 mm lateral to the midline); additional leads were placed in the nuchal muscle for EMG recording.

2.3. Experimental Design

2.3.1. Compounds—Dose ranges were chosen that previously produced APD-like activity in preclinical rodent models. (Ahnaou et al., 2003; Bubser et al., 2014; Maehara et

al., 2008; Stanhope et al., 2001) VU0467154 and xanomeline L-tartrate were synthesized in-house (Bubser et al., 2014) and formulated in 10% Tween 80 as an aqueous microsuspension and solution, respectively. Clozapine and (+)MK-801 hydrogen maleate (both Sigma-Aldrich, MO) were dissolved in 1% glacial acetic acid in sterile water and saline, respectively. All compounds' formulations were adjusted to pH 6-7 and dosed intraperitoneally (i.p.) at 2 mL/kg (VU0467154) or subcutaneously (s.c) at 1 mL/kg (xanomeline, clozapine, and MK-801). Compound administration followed a counter-balanced design for each compound tested with a minimum of 5 days (washout) between doses (n=8-12 rats/dose group; separate vehicle groups were tested for each compound).

2.3.2. Sleep architecture and arousal—To examine wake-promoting or sleep-altering effects, VU0467154, clozapine and xanomeline were administered 2 h after light onset, when rats are predominately sleeping. To examine sleep-promoting or sedative effects, the top dose of each compound was administered 2 h after light offset when rats are predominately awake and active.

2.3.3. MK-801-induced elevations in gamma power—To examine antipsychotic-like activity in an acute model of NMDAR hypofunction, the top dose of each compound was administered 30 minutes prior to administration of 0.18 mg/kg MK-801, coinciding with 2.0 and 2.5 h after light onset. Selection of the MK-801 dose was based on previous studies demonstrating robust elevations in gamma power (Hiyoshi et al., 2014; Kocsis, 2012).

2.4. Data Collection and Analysis

2.4.1. Sleep/Wake Staging—EEG and EMG data were collected using Dataquest A.R.T. 4.3 software (DSI) using a continuous sampling rate of 500 Hz. Blinded observers manually scored each 10-sec epoch using Neuroscore 3.0 software as awake, NREM or REM sleep based on accepted characteristic oscillatory patterns (Brown et al., 2012; Ivarsson et al., 2005). See Supplemental Figure S1A for representative EEG and EMG traces. 10-sec epochs were summed into 60-min bins to examine the percent of time spent awake/h for each 24-h period. NREM and REM sleep latency were determined, defined as the first >30-sec bout of sleep following dosing or the first >20-sec bout of REM sleep following NREM sleep onset, respectively. The cumulative amount of total sleep, NREM and REM sleep were also examined over a 24-h period.

2.4.2. qEEG Spectral Power Analysis—State-dependent relative power spectra from frontal, parietal, and occipital electrodes were computed in 1-Hz bins from 0.5 to 100 Hz using a Fast Fourier Transformation and calculated as a percent of total power as previously described (Nedelcovych et al., 2015). See Supplemental Methods for more details. Specifically, we examined the pharmacological effects on arousal only during epoch that rats were awake. The power spectrum 1-2 h post dosing was expressed as the percent change within each respective 1-Hz interval from the 1 h interval prior to dosing (baseline). qEEG changes are discussed in terms of power bands according to convention as Delta (0.5-4 Hz), Theta (4-8 Hz), Alpha (8-13 Hz), Beta (13-30 Hz), Low Gamma (30-50 Hz), and High Gamma (50-100 Hz) (Brown et al., 2012; Nedelcovych et al., 2015).

In addition, we examined variables known to be disrupted in schizophrenia, or that are altered by APDs, specifically high gamma power during wake and slow wave activity during NREM sleep. To examine these time-dependent effects on qEEG measures, power within a discrete state and frequency band was plotted across time. Changes in high frequency gamma power (50-100 Hz) during the wake state were averaged in 10-min bins and expressed as a percent change from a 1-h baseline period prior to compound administration alone or in combination with MK-801. We also examined the average percent change in high gamma power from 60-240 minutes after MK-801 administration. Lastly, slow wave activity (SWA) defined as delta power (0.5-4 Hz) in the frontal cortex during NREM sleep was calculated in 1-h bins and expressed as a percent change from the averaged delta power during the 1-h baseline period prior to dosing. Individual data points for SWA were excluded if less than 5 min of NREM sleep occurred in any 1-h bin. qEEG was not examined during REM sleep as all compounds reduced REM sleep duration, minimizing the amount of data for comparison within relevant timeframes.

To provide further information regarding pharmacokinetic-pharmacodynamic interactions, plasma concentrations were collected following VU0467154 and xanomeline administration. Additionally, a modified Irwin screen was conducted to assess potential adverse effects of the highest dose of each compound (VU0467154 and xanomeline, 30 mg/kg; clozapine, 10 mg/kg) on motor and autonomic function. See Supplemental Methods for description of these studies.

2.4.3. Statistical analysis—24-h assessments of percent time awake were examined using two-way repeated measure analysis of variance (2-WAY RM ANOVA) assessing effects of time (repeated measure) and dose; non-repeated 2-WAY ANOVA were applied to assess SWA due to missing time points (see above). To examine NREM and REM latencies, and cumulative sleep durations, one-way ANOVAs were applied. To examine time- and dose-dependent effects on high gamma power during wake alone and following MK-801 administration, and to examine SWA during NREM sleep, 2-WAY ANOVAs were applied. Additionally, a one-way ANOVA was applied to compare effects of VU0467154, clozapine or xanomeline on average percent change in high gamma power across the 60-240 minutes following the initial compound administration. If significant, main effects of time-dependent analyses were followed by Bonferroni's *post hoc* tests comparing each compound to respective vehicle-treated groups. For all experiments, significance was defined as $p < 0.05$. To examine effects of dose and frequency across the full power spectra (0.5-100 Hz) within a discrete time point (1-2 h), 2-WAY ANOVAs were applied and if significant, followed by a Fisher's LSD *post hoc* test with significance defined as $p < 0.05$ (Leiser et al., 2014).

3. Results

3.1. VU0467154 increases arousal during wake but does not promote wakefulness

Consistent with the nocturnal nature of rodents, ~70% of time during the light phase was spent sleeping and ~70% of time during the dark phase was spent awake (Figure 1). Consistent with the literature, compound administration 2 h into the light cycle resulted in a transient increase in time awake that dissipated within ~30 min following vehicle

administration. There was a significant effect of VU0467154 on percent time awake across the 24-h period (Fig 1A; dose [$F_{3,42}=3.14$, $p<0.05$], time [$F_{23,966}=50.20$, $p<0.0001$] and dose $\times$ time interaction [$F_{69,966}=1.36$, $p<0.05$]), yet no dose of VU0467154 was different from vehicle at any time point. Clozapine affected percent time awake in a dose- and time-dependent manner (Fig 1B; dose [$F_{3,35}=5.36$, $p<0.01$], time [$F_{23,805}=27.77$, $p<0.0001$] and dose $\times$ time interaction [$F_{69,805}=p<0.0001$]) such that 3 mg/kg clozapine produced a significant increase at early time points and 10 mg/kg clozapine produced a significant decrease during the dark period; the long-lasting effects are not consistent with the pharmacokinetic profile of clozapine (half-life ~ 100 min; Baldessarini et al., 1993). The M_1/M_4 mAChR agonist xanomeline dose-dependently increased percent time awake (Fig 1C; dose [$F_{3,43}=11.77$, $p<0.0001$], time [$F_{23,989}=46.45$, $p<0.0001$] and dose $\times$ time interaction [$F_{69,989}=7.33$, $p<0.0001$]).

To compare effects of VU0467154 on arousal with clozapine and xanomeline, we examined relative spectral power specifically during epochs scored as awake at 1-2 h post dosing. This time period coincided with previously reported antipsychotic-like and/or cognition enhancing activity for all three compounds (Bubser et al., 2014; Hiyoshi et al., 2014; Stanhope et al., 2001). As shown in Figure 1D, 10 and 30 mg/kg VU0467154 (blue, green horizontal lines, respectively) decreased power in alpha and low beta bands and increased power in the high gamma band in the frontal cortex (dose [$F_{3,42}=3.46$, $p<0.05$], frequency [$F_{100,4200}=10.35$, $p<0.0001$] and dose $\times$ frequency interaction [$F_{300,4200}=2.95$, $p<0.0001$]). In contrast to VU0467154, clozapine increased theta, and alpha and dose-dependently decreased gamma power in the frontal cortex (Figure 1E; dose [$F_{3,36}=3.87$, $p<0.05$], frequency [$F_{100,3600}=21.75$, $p<0.0001$] and dose $\times$ frequency interaction [$F_{300,3600}=6.26$, $p<0.0001$]). In addition, 1 mg/kg clozapine (red line) decreased alpha and increased high gamma power. As shown in Figure 1F, similar to VU0467154 3 and 10 mg/kg xanomeline (red, blue lines respectively) increased low frequency delta power, 10 and 30 mg/kg xanomeline (green lines) decreased alpha and low beta power and all three doses increased power in the high gamma frequency band in a dose-dependent manner in the frontal cortex (frequency [$F_{100,4100}=54.82$, $p<0.0001$] and dose $\times$ frequency interaction [$F_{300,4100}=4.07$, $p<0.0001$]). Effects on spectral power were similar in parietal and occipital regions for all three compounds although the magnitude of effects varied (See **Supplemental Figure S2**).

In addition, 10 mg/kg clozapine, but not the top doses of VU0467154 or xanomeline decreased motoric function when dosed during the light period (e.g. catalepsy, leg, weakness, grasping loss) as determined by the Modified Irwin Neurological Screen (See **Supplemental Figure S3**). Lastly, time course analysis showed that VU0467154 and xanomeline elevated high frequency gamma power by $\sim 25\%$ and 50% , respectively and clozapine reduced gamma power by $\sim 25\%$ (see **Supplemental Figure S3**).

3.2. VU0467154 selectively increases REM sleep latency and increases total and NREM sleep

To understand the role of M_4 on initiation of sleep states, we examined latency to enter NREM and REM sleep. 30 mg/kg VU0467154 selectively increased REM sleep latency without altering NREM sleep latency, similar to effects of clozapine (see Table 1). In

contrast, xanomeline significantly increased both NREM and REM sleep latency, consistent with wake-promotion (all $p < 0.05$).

In addition, we examined cumulative sleep duration across the 24-h period following compound administration 2 h after light onset. 30 mg/kg VU0467154 increased total sleep and NREM sleep across the 24-h period compared to vehicle-treated rats (Table 1). Similar to VU0467154, 10 mg/kg clozapine increased NREM sleep duration. Clozapine had a significant effect on total sleep and REM sleep duration (increase and decrease, respectively) yet no doses were different from vehicle-treated rats. Xanomeline significantly decreased total and NREM sleep durations at the 10 and 30 mg/kg dose and REM sleep duration at the 30 mg/kg dose across the 24-h period compared to vehicle-treated rats. See **Supplemental Figure S4**, for cumulative sleep duration compared to vehicle-treated rats in 1-hr bins. Because VU0467154 plasma concentrations were still elevated 24h post-injection, the sustained effects of VU0467154 over 24 h were likely pharmacodynamic in nature and not a rebound sleep (see **Supplemental Figure S5 and Table S1**).

3.3 VU0467154 does not disrupt slow wave activity during NREM sleep

To examine the effects of selective activation of M_4 by VU0467154 on sleep quality, we assessed slow wave activity (SWA; delta power) during NREM sleep, a neurophysiological marker of sleep quality (Cohrs, 2008; Steiger and Kimura, 2010). As shown in Figure 2, in vehicle-treated rats SWA decreased across the 12-h light period, consistent with decreased sleep drive associated with prevalence of sleep during this period (Nedelcovich et al., 2015; Vyazovskiy et al., 2011). Across the 24-h period, <3% of time points were excluded from analysis due to insufficient sleep duration for VU0467154 and clozapine (<5 min/h; 27 points [VU0467154] and 20 points [clozapine]). As shown in Figure 2A, there was an overall effect of VU0467154 on SWA (dose [$F_{3,932}=14.02$, $p < 0.0001$], time [$F_{23,932}=8.12$, $p < 0.0001$] but no dose $\times$ time interaction) yet no time points were different from vehicle-treated rats. As shown in Figure 2B, Clozapine increased SWA (dose [$F_{3,868}=29.10$, $p < 0.0001$] and dose $\times$ time interaction [$F_{69,868}=1.51$, $p < 0.01$]). Due to the wake-promoting effects of xanomeline, 120 points were excluded due to insufficient sleep duration (~10%), occurring in the first hours post dosing. In addition, only 1 rat dosed with 10 and 30 mg/kg xanomeline entered NREM sleep during the 1-2 and 2-3 h time point post dosing and were also excluded from statistical analysis. As shown in Figure 2C, xanomeline decreased SWA compared to vehicle-treated rats (dose [$F_{3,859}=44.03$, $p < 0.0001$], time [$F_{21,859}=14.18$, $p < 0.0001$] and dose $\times$ time interaction [$F_{63,859}=1.70$, $p < 0.001$]).

3.4. VU0467154 does not alter percent time awake or arousal when dosed during the dark period

Sleep architecture, baseline arousal levels (see **Supplemental Figure S7**) and acetylcholine levels fluctuate across the diurnal cycle (Hut and Van der Zee, 2011; Kikuchi et al., 2013; Platt and Riedel, 2011; Takase et al., 2009; Vyazovskiy et al., 2011). Thus, examining drug effects at different time points across the 24-h period is critical to understand potential therapeutic index, especially with regard to allosteric modulation. Because VU0467154 increased total and NREM sleep duration across the 24 h period, we wanted to directly assess potential sleep-promoting effects or sedative effects on arousal states. A single high

dose of VU0467154 (30 mg/kg), xanomeline (30 mg/kg), and clozapine (10 mg/kg) was administered 2 h after light offset during the dark phase. As shown in Figure 3A, VU0467154 did not alter percent time awake (significant effect of time [$F_{23,460}=33.52$, $p<0.0001$] but not dose or dose $\times$ time interaction). Clozapine did not alter percent time awake (Figure 3B; significant effect of time [$F_{23,437}=22.53$, $p<0.0001$] but not dose or dose $\times$ time interaction). Xanomeline significantly increased percent time awake (Figure 3C; dose [$F_{1,20}=280.006$, $p<0.0001$], time [$F_{23,460}=56.24$, $p<0.0001$] and dose $\times$ time interaction [$F_{23,460}=8.28$, $p<0.0001$]). SWA and sleep onset were not examined following dosing in the dark period as levels of sleep were low during this period.

As shown in Figure 3D, VU0467154 did not affect spectral power in the frontal cortex during waking epochs only, 1-2 h after dosing in the dark period (significant effect of frequency [$F_{100,2000}=3.51$, $p<0.001$] but not dose or dose $\times$ frequency interaction). These data are distinct from the effects of VU0467154 when dosed during the light period. In contrast, clozapine and xanomeline produced similar effects on power spectra as when dosed during the light period. Clozapine increased theta, alpha and low beta power and decreased low gamma power (Figure 3E; dose [$F_{1,20}=6.50$, $p<0.05$], frequency [$F_{100,2000}=11.13$, $p<0.0001$] and dose $\times$ frequency interaction [$F_{100,2000}=18.77$, $p<0.0001$]). Xanomeline decreased alpha power and increased high gamma power (Figure 3F; dose [$F_{100,2000}=8.13$, $p<0.0001$] and dose $\times$ treatment interaction [$F_{100,2000}=3.19$, $p<0.001$] but not an effect of treatment).

3.5. VU0467154 attenuates MK-801-induced elevations in high gamma frequency power

To extend previous studies demonstrating that selective activation of M_4 by VU0467154 reversed MK-801-induced cognitive disruptions and hyperlocomotion (Bubser et al., 2014), we examined the ability of VU0467154 to attenuate MK-801-induced neurophysiological alterations in high frequency gamma power, a potential biomarker of NMDAR hypofunction (Hiyoshi et al., 2014; Kocsis, 2012). As shown in Figure 4 and similar to previous reports (Hiyoshi et al., 2014; Kocsis, 2012), MK-801 increased high frequency gamma power by ~100% in the frontal and parietal cortices (Figure 4A and C) and ~150% in the occipital cortex (Figure 4E). One rat was excluded from all groups because MK-801 alone failed to increase gamma power by >25% in the occipital cortex. Pretreatment with a single high dose of VU0467154 (30 mg/kg), xanomeline (30 mg/kg), and clozapine (10 mg/kg) altered MK-801-induced gamma power in the frontal cortex (Figure 4A; dose [$F_{3,1626}=256.90$, $p<0.0001$], time [$F_{41,1626}=9.45$, $p<0.0001$] and dose $\times$ time interaction [$F_{123,1626}=2.29$, $p<0.0001$]), parietal cortex (Figure 4C; dose [$F_{3,1624}=587.20$, $p<0.0001$], time [$F_{41,1624}=20.20$, $p<0.0001$] and dose $\times$ time interaction [$F_{123,1624}=5.25$, $p<0.0001$]), and occipital cortex (Figure 4E; dose [$F_{3,1622}=288.30$, $p<0.0001$], time [$F_{41,1622}=48.00$, $p<0.0001$] and dose $\times$ time interaction [$F_{123,1622}=3.25$, $p<0.0001$]). As shown in Figure 4C (indicated by light gray horizontal bar), pretreatment with VU0467154 attenuated MK-801 induced elevations in gamma power in the parietal cortex. Consistent with previous studies (Hiyoshi et al., 2014), clozapine significantly decreased gamma power in all three regions (Figure 4A, C, and E) prior to and following MK-801 administration compared to the vehicle-MK-801-treated control group. In contrast, xanomeline increased gamma power in the parietal cortex and to a lesser degree in the occipital cortex (Figure 4C). There was a

significant effect of treatment on MK-801-induced elevations on average gamma power in the 60-240 minutes after initial compound administration in the frontal ($F_{3,39} = 8.97$; $p < 0.0001$), parietal ($F_{3,39} = 31.67$) and occipital ($F_{3,39} = 16.31$) cortex. As shown in Figure 4D, pretreatment with VU0467154 reduced gamma power across the 60-240 min period in parietal cortex ($p < 0.05$). Clozapine significantly reduced average percent change in gamma power from 60-240 minutes after initial compound administration in the frontal, parietal and occipital cortex (Figures 4B, D, F, respectively, all $p < 0.001$).

4. Discussion

Discovery and characterization of the M_4 PAM VU0467154 represents a critical breakthrough in the development of subtype-selective mAChR ligands that can enhance and maintain activity-dependent signaling at the M_4 mAChR for the potential treatment of the complex symptoms of schizophrenia, including disruptions in sleep architecture, arousal and cognition. Here, we examined the potential impact of selective allosteric potentiation of M_4 on state-dependent alterations on sleep architecture, arousal and APD-like activity. We have chosen to focus our analysis of this large data set on the following specific variables, wake-promoting or sleep-altering effects (REM/NREM sleep duration, sleep quality) and arousal during wake since these measures are disrupted in patients with schizophrenia or are directly affected by APDs (in a therapeutic or adverse manner). We also examined whether a selective M_4 PAM can reverse MK-801-induced changes in high gamma power, which represents a translatable measure of a neurophysiological disturbance associated with schizophrenia. Interestingly, the M_4 PAM VU0467154 altered both sleep and wake states consistent with potential therapeutic effects in contrast to orthosteric mAChR agonists or APDs that only address symptoms in one state and/or display adverse side effects in other states. VU0467154 also reversed MK-801-induced elevations in gamma power in a brain region-dependent manner. Importantly, the doses of VU0467154 that produced changes in qEEG in the present study are associated with improved cognitive performance alone and in pharmacologically-induced disruption models of the cognitive impairments associated with schizophrenia (Bubser et al., 2014).

Accumulating evidence suggests that normalizing aberrant sleep architecture associated with schizophrenia may improve multiple symptom clusters, prevent further symptom onset, and improve quality of life (Cohrs, 2008; Manoach and Stickgold, 2009; Pritchett et al., 2012; Sprecher et al., 2015). In general, APDs alleviate some of the sleep disruptions in patients with schizophrenia by increasing sleep duration, delaying REM onset, increasing the amount of SWS compared to REM sleep, and/or increasing SWA during SWS (Cohrs, 2008; Krystal et al., 2008; Sprecher et al., 2015). However, the effects of clozapine and other APDs on sleep do not substantially improve cognition (Barch and Ceaser, 2012; Nuechterlein et al., 2004), perhaps due to residual sedative effects during wake. Daytime drowsiness or decreased arousal is common following APD administration in healthy humans, schizophrenia patients, and in healthy rats (Ahnaou et al., 2003; Czobor and Volavka, 1993; Dimpfel, 2007; Hughes et al., 1999; Roubicek and Major, 1977; Wichniak et al., 2006; Yoshimura et al., 2007). In the present studies, VU0467154 altered sleep in a manner similar to clozapine, suggesting potential APD-like activity on sleep architecture, but lacked the sedative profile of clozapine during waking periods. Similarly, the profile of VU0467154 is

distinct from the effects of current sleep aids in both rodents and healthy humans in that hypnotics promote sleep without improving aspects of sleep architecture (Brunner et al., 1991; Fox et al., 2013) and are associated with detrimental effects on cognition in the morning following exposure (Stranks and Crowe, 2014). Further, studies examining benzodiazepines and newer “z-drugs” in patients with schizophrenia have largely shown negative results, including impaired sleep and cognition (for review see, Kantrowitz et al., 2009).

Quantitative EEG studies have revealed abnormal brain oscillation patterns in unmedicated schizophrenia patients at rest and in response to cognitive tasks. qEEG can serve as a neurophysiological marker for arousal or vigilance states during both sleep and wake periods that can indicate therapeutic or adverse effects. For example, SWA during SWS provides a marker of sleep quality. During wake, qEEG can assess vigilance along a continuum of sedate (e.g. hypoarousal, elevated power in low frequencies) to alert (e.g. cortical activation, increased high frequency power) to hyper-aroused (e.g. excessive high power in gamma frequencies) (Buzsaki and Silva, 2012; Graef et al., 2011). Alpha-(8-12 Hz) activity has been inversely correlated with levels of arousal and attentional selection (Barry et al., 2007; Busch et al., 2009; Mazaheri et al., 2009), while increased gamma (30-100 Hz) activity is necessary for cognitive performance (Buzsaki and Silva, 2012). During waking intervals of the light period when baseline gamma power was relatively low, VU0467154 altered power in a manner similar to xanomeline and consistent with enhanced arousal. However, VU0467154 did not alter the power spectra when administered during the dark period while rodents are most alert, consistent with a lack of potential sedating or hyper-arousing effects of the M₄ PAM mechanism. In contrast, clozapine increased low, and decreased high power, consistent with the sedative effects of APDs in medicated patients with schizophrenia (Boutros et al., 2008; 2014; Goder et al., 2006; Keshavan et al., 1995a; Wichniak et al., 2006). Clozapine and xanomeline administration produced similar effects, regardless of dosing during the light or dark period.

Our findings suggest that selective M₄ mAChR potentiation may improve sleep disturbances, enhance arousal during wake, and lack a sedative profile commonly associated with APDs. This state-dependent profile is distinct from xanomeline, a compound that may improve daytime functions yet impairs sleep quality, and clozapine, which may improve sleep architecture yet induces drowsiness during waking periods. Although additional studies are warranted, M₄ potentiation may contribute to acute or long-term improvements in cognitive and functional outcomes in patients with schizophrenia. State-dependent effects of VU0467154 may be due to fluctuations in cholinergic tone across the diurnal cycle that parallel changes in arousal states and sleep propensity. Specifically, acetylcholine (ACh) levels are elevated during wake and REM sleep and decreased during non-REM (NREM) sleep (Crouzier et al., 2006; Williams et al., 1994). Future studies will examine specific effects of M₄ mAChR potentiation on ACh levels across the diurnal cycle as well as evaluate acute versus repeated dosing with VU0467154 and additional M₄ PAMs with distinct chemotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from the National Institute of Mental Health (MH086601, C.K.J.; MH073676, MH087965, MH093366, P.J.C.), by AstraZeneca and from a PhRMA Foundation postdoctoral fellowship in Pharmacology and Toxicology (RWG). We thank Dina McGinnis and Weimin Peng for surgical expertise, Dr. Ariel Deutch and Dr. David Devilbiss for helpful comments on data analysis and interpretation, and Frank Byers for conducting the in-life phase of the pharmacokinetic studies. All pharmacodynamic studies were performed within the Rat and Murine Neurobehavioral Laboratories at Vanderbilt University Medical Center.

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Highlights

- Sleep disturbances are associated with primary symptoms in schizophrenia.
- Antipsychotics (APDs; clozapine) may improve sleep but cause sedation during wake.
- M₄ muscarinic acetylcholine receptors (mAChR) represent a novel target for APDs.
- VU0467154 is a potent M₄ mAChR positive allosteric modulator with APD-like effects.
- VU0467154 alters sleep like clozapine, without a sedative profile during wake.

Figure 1. VU0467154 increases arousal during wake but does not promote wakefulness

A-C, percent time awake in 1-h epochs ($\pm$ SEM) across the 24-h period when dosed 2 h into the light period; open symbols, $p < 0.05$ vs respective vehicle-treated rats. Vertical gray bars in A-C denote the 1-2 hr period post dosing in which spectral power was examined in panels D-F. D-F, changes in relative spectral power in the frontal cortex (% change from baseline, BL) during waking epochs only, during the 1-2 h period following VU0467154 (D), Clozapine (E) and Xanomeline (F) administration. Relative power is summed in 1 Hz bins (0.5-100 Hz) from all 10-sec waking epochs and expressed as a percent change ($\pm$ SEM) from respective power within the same frequency bin during waking epochs from the 1 h baseline (BL) period prior to dosing. Gray/tan vertical bars represent frequency bands (Δ , delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-100 Hz). Corresponding colored horizontal dots/lines represent frequencies at each dose that were statistically different from vehicle treated rats ($p < 0.05$, LSD *post hoc*), $n = 9-12/\text{group}$.

Figure 2. VU0467154 does not disrupt slow wave activity during NREM sleep
 VU0467154 (A), Clozapine (B) and Xanomeline (C), were administered 2 h into the light period. Slow Wave Activity (SWA), defined as delta power during NREM sleep, is expressed as % change ($\pm$ SEM) from 1 h pre-dosing baseline (BL). Filled black symbols, $p < 0.05$ compared to vehicle-treated rats, Bonferroni *post hoc*, $n = 9-12$ /group.

Neuropharmacology. Author manuscript; available in PMC 2017 March 01.

Figure 4. VU0467154 attenuates MK-801-induced elevations in high gamma frequency power
 The time course of MK-801-induced elevations (black, circles) in high frequency gamma power (50-100 Hz) and attenuation by 30 mg/kg VU0467154 (VU154, squares), 10 mg/kg Clozapine (CLZ upward triangles), and lack of effect of 30 mg/kg xanomeline (XAN, downward triangles) is shown for EEG recordings from (A) frontal, (C) parietal, and (E) occipital cortices. Data are shown in 10 minute bins expressed as percent change from averaged 60 minute baseline (BL) during waking epochs only. Average percent change from baseline across 60-240 min period following initial compound administration are shown for

(B) frontal, (D) parietal, and (F) occipital cortices. Corresponding colored horizontal lines represent time points that were statistically different from vehicle+ MK-801-treated rats. lines, $p<0.05$, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$, Bonferroni *post hoc*, $n=9-11$ /group.

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Table 1

VU0467154 increases total and NREM sleep duration and delays REM sleep onset.

Dose (mg/kg)	Sleep Latency [Min (SEM)]		24 h Sleep Duration [Min(SEM)]		
	NREM	REM	TST	NREM	REM
VU0467154					
1-WAY ANOVA	F _{3,42} =0.76	F _{3,42} =5.68 [^]	F _{3,42} =3.13 [*]	F _{3,42} =2.99 [*]	F _{3,42} =0.20
Veh	31.71 (4.17)	24.67 (4.19)	766.43 (16.66)	658.33 (16.30)	104.50 (5.25)
3	26.68 (4.68)	35.82 (6.71)	774.01 (13.40)	663.94 (16.85)	100.83 (4.08)
10	30.25 (4.76)	52.71 (8.40)	784.69 (13.08)	675.05 (13.63)	100.49 (3.17)
30	38.59 (6.80)	82.14 (18.88) [^]	828.20 (20.85) [*]	722.74 (22.53) [*]	100.96 (4.96)
Ciozapine					
1-WAY ANOVA	F _{3,35} =2.33	F _{3,35} =60.32 ⁺	F _{3,35} =4.75 [*]	F _{3,35} =6.06 [^]	F _{3,35} =3.78 [*]
Veh	29.04 (5.77)	30.24 (4.24)	790.66 (29.86)	658.20 (32.92)	124.22 (3.39)
1	18.33 (3.25)	104.69 (19.91)	760.85 (20.16)	643.6 (22.61)	115.76 (6.48)
3	18.03 (3.26)	223.57 (19.47) [#]	759.13 (24.71)	622.92 (26.03)	128.23 (6.96)
10	15.21 (3.85)	476.64 (40.38) [#]	874.67 (22.6)	759.46 (22.79) [*]	103.45 (6.04)
Xanomeline					
1-WAY ANOVA	F _{3,43} =17.27 ⁺	F _{3,43} =13.58 ⁺	F _{3,43} =11.77 ⁺	F _{3,43} =9.28 ⁺	F _{3,43} =7.62 [#]
Veh	36.90 (12.05)	28.17 (47.40)	795.45 (24.41)	688.89 (19.25)	104.93 (9.36)
3	79.79 (13.07)	79.88 (14.48)	766.04 (11.84)	670.15 (15.02)	94.65 (6.43)
10	197.94 (22.79) [#]	140.83 (28.30)	662.29 (31.71) [^]	566.21 (27.29) [^]	94.81 (7.39)
30	234.28 (37.23) [#]	394.46 (84.08) [#]	582.32 (43.76) ⁺	523.58 (42.57) [#]	57.30 (7.60) [#]

NREM sleep onset defined as 1st epoch of NREM sleep lasting >30 sec

REM sleep onset defined as first REM sleep epoch >20 sec following from NREM sleep onset

* p <0.05

^ p <0.01

# p < 0.001

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