Selectively Targeting the M_1 and M_4 Muscarinic Acetylcholine Receptors for the Treatment of Age-Related Sleep/Wake Architecture and Arousal Deficits

By

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CHAPTER 1

Introduction

1.1. The Cholinergic System

The molecule acetylcholine (ACh) was first discovered in 1914 (Ewins, 1914) and was the first neurotransmitter identified in mammalian systems in 1921 (H. Ferreira-Vieira et al., 2016; Loewi, 1921). ACh acts in both the peripheral and central cholinergic systems. In the central nervous system, ACh has been implicated in numerous cognitive functions such as memory, including working and long-term spatial memory (Solari and Hangya, 2018), cue detection (Sarter et al., 2014), and attentional processes (Klinkenberg et al., 2011) (For complete discussion, see section 1.1.3.). Central cholinergic signaling is also vital for modulating sleep/wake states and arousal (B. E. Jones, 2020) (For complete discussion, see section 1.1.4.). Beyond cognition, sleep/wake architecture and arousal the central cholinergic system regulates motor function, food intake, addictive behaviors, and nociception (Kini, 2019). Acetylcholine is vital in the autonomic nervous system, where activation of the peripheral cholinergic system is responsible for the induction of salivation, bladder motility, gastrointestinal motility, ocular functions, and modulation of heart rate and cardiac contractility (Abrams et al., 2006). Additionally, ACh is the primary transmitter at the neuromuscular junction (Nishimune and Shigemoto, 2018). This dissertation will focus on the assessment of selective small molecule ligands for the modulation of the various functions within the central cholinergic system. Specifically, how these cholinergic ligands impact sleep/wake architecture and arousal in young and non-pathologically aged mice, where non-pathologically aged mice are defined wildtype mice that have aged with no pathological abnormalities. It will also be important to consider the effects of these ligands on the peripheral cholinergic system when assessing associated peripheral adverse effects.

1.1.1. Central Cholinergic System Anatomy

The central cholinergic system consists of eight nuclei (Ch1-Ch8) (M. M. Mesulam, 1990). Of these eight nuclei, four make up the cholinergic forebrain nuclei: the medial septum (Ch1), the vertical limb of the diagonal band of Broca (Ch2), the horizontal limb of the diagonal band of Broca (Ch3), and the nucleus basalis of Meynert (Ch4). Two cholinergic nuclei are found within the brainstem: the pedunculopontine nucleus (PPN) (Ch5) and the laterodorsal tegmental nucleus (LDT) (Ch6) (Figure 1.1). The two remaining nuclei are the medial habenula (Ch7) and the parabigeminal nucleus (Ch8) (M. M. Mesulam, 1990; M. Marsel Mesulam et al., 1983) (Figure 1.1).

The cholinergic forebrain nuclei provide the major afferent innervation for neocortical and limbic regions, with the vertical and horizontal limbs of the diagonal band of Broca and the nucleus basalis of Meynert providing significant projections to cortical areas (Ährlund-Richter et al., 2019; Dautan et al., 2016; Rye et al., 1984; Waterhouse and Chandler, 2012) (Figure 1.1),

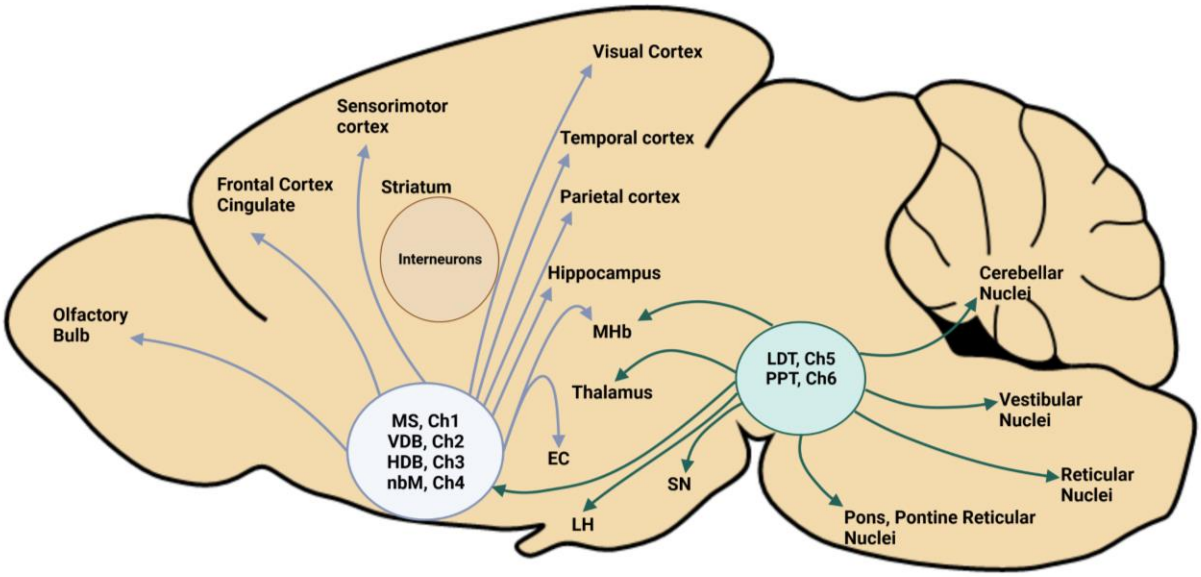

Figure 1.1. Summary of major central cholinergic mammalian projections. Abbreviations, EC: entorhinal cortex, HDB: horizontal band of the diagonal band of Broca, LDT: laterodorsal tegmental nucleus, LH: lateral hypothalamus, MHb: medial habenula, MS: medial septum, nbM: nucleus basalis of Meynert, PPT: pedunculopontine tegmental nucleus, SN: substantia nigra, VDB: vertical band of the diagonal band of Broca. Created with BioRender.com.

while projections from the medial septum have been described to the medial prefrontal cortex (Ährlund-Richter et al., 2019) and the cingulate cortex (Dautan et al., 2016) (Figure 1.1), and minor projections from the PPT to cortical areas are observed (Dautan et al., 2016; Satoh and Fibiger, 1986). Furthermore, the medial septum provides rich cholinergic innervation to hippocampal areas (Dautan et al., 2016; Rye et al., 1984), with projections to the hippocampus from the vertical limb of the diagonal band of Broca (Dautan et al., 2016; Rye et al., 1984) and the horizontal limb of the diagonal band of Broca also described (Dautan et al., 2016) (Figure 1.1). The brainstem cholinergic nuclei (PPT and LDT) provide substantial innervation to thalamic areas (Dautan et al., 2016; Satoh and Fibiger, 1986; Sofroniew et al., 1985; Sokhadze et al., 2022), with projections to the nucleus accumbans, cholinergic forebrain nuclei, striatal structures, and other minor projections also observed (Dautan et al., 2016; Satoh and Fibiger, 1986) (Figure 1.1). Projections from the medial habenula target the interpeduncular nucleus (Figure 1.1), and projections from the parabigeminal nucleus target the inferior and superior colliculi (Dautan et al., 2016).

1.1.2. The Cholinergic Synapse and Receptors

ACh is synthesized in the presynaptic terminal by the enzyme choline acetyltransferase (ChAT), which combines choline and acetyl coenzyme A (D. Wu and Hersh, 1994). The vesicular ACh transporter (vAChT) then packages the ACh in vesicles ready for release (Eiden, 1998). Within the synaptic cleft, the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are responsible for the degradation of ACh by hydrolysis of ACh to acetate and choline (Darvesh et al., 2003; Trevor et al., 1978). Choline is then recycled into the presynaptic terminal through the high-affinity choline transporter (CHT) in the rate-limiting step for acetylcholine synthesis (Ferguson et al., 2003).

There are two different families of receptors activated by ACh, the nicotinic (nAChRs) (Dani, 2001) and muscarinic acetylcholine receptors (mAChRs) (Bubser et al., 2012). nAChRs are a

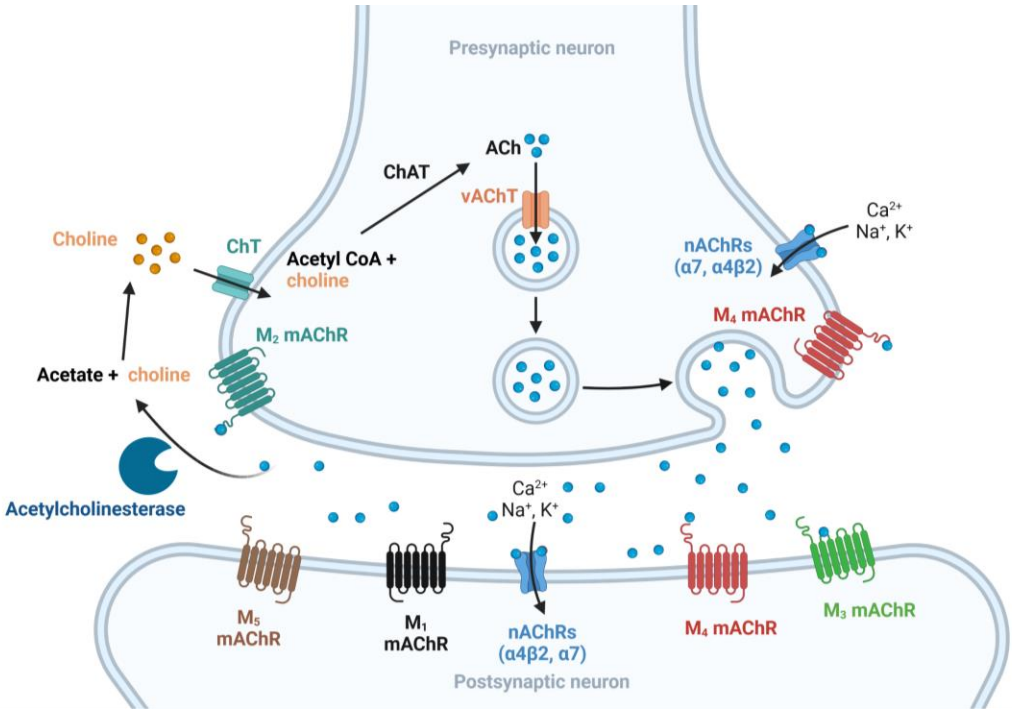

Figure 1.2. The Cholinergic Synapse. Abbreviations: Acetyl-CoA: acetyl coenzyme A, ChAT: Choline Acetyltransferase, VAChT: Vesicular Acetylcholine Transporter, ChT: High-affinity Choline Transporter. AChE: Acetylcholinesterase, nAChR: Nicotinic acetylcholine receptors, mAChR: Muscarinic acetylcholine receptors. Created with BioRender.com

family of pentameric ligand-gated ion channels, of which the α_7- and $\alpha_4\beta_2$-containing nAChRs are the predominant subtypes found in the central nervous system (Seguela et al., 1993; Wada et al., 1989). nAChRs are ideally placed to modulate cognition and arousal with $\alpha_4\beta_2$-containing nAChRs found in the cortex, hippocampus, striatum, thalamus, amygdala, substantia nigra, and numerous hindbrain nuclei, and α_7-containing nAChRs found in similar areas except for the thalamus (Gotti et al., 2006). In fact, numerous studies report the beneficial effects of nicotinic activation on cognitive performance. In preclinical rodent and non-human primate species, selective α_7 and/or $\alpha_4\beta_2$ nAChR ligands have been suggested to enhance memory and/or attentional functions (Azimi et al., 2020; Buccafusco and Terry, 2009; McLean et al., 2011; Wallace et al., 2011), with nAChR agonists producing similar effects on attention and memory in clinical populations (le Houezec et al., 1994; Mancuso et al., 1999; P. A. Newhouse et al., 2004; Potter and Newhouse, 2008). Although these studies suggest a critical role for the nicotinic system in cognition under normal

conditions and in non-pathological aging and Alzheimer's Disease (AD), further discussion of these topics is beyond the scope of this thesis. The mAChRs are G-protein coupled receptors, of which there are five different mAChR subtypes (M_1-M_5). M_1, M_3, and M_5 are expressed post-synaptically and signal through Gq/G11- type G-proteins leading to activation of several signal transduction cascades, including activation of phosphoinositide-specific phospholipase-Cβ leading to the production of inositol-1,4,5-triphosphate and 1,2-diacylglycerol and subsequent increases in intracellular calcium (Florio and Sternweis, 1985; Shaw and Exton, 1992). A critical function of the M_1 receptor is that it physically and functionally couples to the glutamatergic N-methyl-aspartate receptor (NMDAR) on glutamatergic pyramidal cells leading to the potentiation of NMDAR currents (Marino et al., 1998). The potentiation of NMDARs in hippocampal circuits leads to enhanced cognitive performance (Cadinu et al., 2018; Marino et al., 1998). Selective activation of M_1 mAChR has been reported to improve several cognitive functions, including memory functions as measured by touchscreen pairwise discrimination, novel object recognition and paired associates learning in rodent and non-human primate species, and executive and attentional functions as measured by the object retrieval detour task and the continuous performance task in non-human primate species (Gould et al., 2015; Lange et al., 2015; Moran et al., 2018; Rook et al., 2018; Vardigan et al., 2015). Both M_2 and M_4 couple through the inhibitory Gi/o G-proteins, inhibiting adenylyl cyclase activity and prolonged opening of potassium and non-selective cation channels (Migeon et al., 1995; Migeon and Nathanson, 1994). The M_2 mAChR is expressed on presynaptic terminals of cholinergic terminals and serves as the predominant autoreceptor for the cholinergic system, acting to reduce ACh release from the presynaptic terminal (Billard et al., 1995; Douglas et al., 2001), while the M_4 mAChR is found pre- and post-synaptically within cholinergic synapses, and as a heteroreceptor at glutamatergic and GABAergic synapses (Foster et al., 2016; Pancani et al., 2014; Tzavara et al., 2003)(Figure 1.2). This unique expression of the M_4 mAChR enables the modulation of multiple neurotransmitter systems. M_4 mAChR activation reduces activity of glutamatergic corticostriatal neurons (Pancani et al., 2014,

2015); decreases cholinergic transmission through its activity as a presynaptic autoreceptor (Tzavara et al., 2003) and reducing striatal dopaminergic activity at D_1 expressing medium spiney neurons in the direct pathway (Foster et al., 2016). This diverse expression enables M_4 mAChR activation to impact an array of behaviors, including cognition, motor movement, and psychotic behaviors (Bubser et al., 2014; Gould et al., 2018; Lange et al., 2021; Moehle et al., 2021).

Neuroanatomical studies utilizing antibodies specific for each of the five mAChR subtypes have revealed a differential expression pattern throughout the CNS (see Teal et al., 2019 for review). This section will focus on expression in areas implicated in sleep/wake architecture, cognition, and arousal. Within the prefrontal cortex, an area richly innervated by the cholinergic forebrain nuclei and important for modulating cognition, M_1, M_2, and M_4 are the most common subtypes, with M_1 seen post-synaptically in pyramidal cells in layers II/III and VI and M_4 detected in the bodies of layer II-IV cells. M_2 has been observed at high levels in terminals at layer IV and the border of layers V and VI (Levey, 1993; Levey et al., 1991, 1995). The hippocampus is also richly innervated by the cholinergic forebrain nuclei and vitally important for normal cognitive function. All the mAChRs, M_1-M_5, have been observed in the hippocampus (Levey et al., 1995; Vilaró et al., 1990). The M_1 and M_3 mAChRs are seen post-synaptically on pyramidal neurons (Levey et al., 1995; Scarr et al., 2016), while M_2 and M_4 mAChR are expressed, at least in part, presynaptically (Levey et al., 1995). M_1-M_4 receptors are found in the striatum (cholinergic interneurons). The hindbrain cholinergic projections project to the thalamus, any area known to modulate sleep-wake architecture (B. E. Jones, 2020). M_1-M_4 mAChRs receptors have been identified within the thalamus (Plummer et al., 1999; Warren et al., 2007; Wei et al., 1994). Overall, the rich expression of M_1 and M_4 receptors in cortical and limbic regions leaves them ideally placed to modulate cognition and arousal under normal conditions and in non-pathological aging and dementia.

1.1.3. Central Cholinergic Systems Role in Cognition

Clinical pharmacological studies in healthy volunteers indicated that the non-selective muscarinic antagonist scopolamine disrupted executive function and memory, highlighting the importance of the muscarinic system in normal cognitive function (Drachman and Leavitt, 1974). These early clinical findings suggest deficits in central cholinergic signaling are responsible, at least in part, for the cognitive deficits observed in non-pathologic aging and dementia populations. More recent clinical work using nicotinic and muscarinic antagonists has suggested that cholinergic function is required for modulating attentional processes (Ellis et al., 2006; P. A. Newhouse et al., 2001; Warburton and Rusted, 1993), performance on memory tasks (Green et al., 2005; Sherman et al., 2003), and executive function (Fredrickson et al., 2008).

Many preclinical studies have supported the clinical findings that nonselective muscarinic cholinergic antagonists impair cognitive function. For example, scopolamine was reported to impair attentional processes as measured by the 5-choice serial reaction time task in rats and non-human primates (Callahan et al., 1993; Jäkälä et al., 1992; D. N. C. Jones and Higgins, 1995). In addition to tasks of attention, the muscarinic antagonist scopolamine produced impairments in spatial memory tasks such as the Morris Water Maze, delayed non-match to sample tasks, and object recognition tasks (Burešová et al., 1986; Dennes and Barnes, 1993; Taffe et al., 1999) and tasks of executive function such as attentional set-shifting (K. C. Chen et al., 2004) (See Terry, 2006 for review of muscarinic antagonist effects on cognition). Furthermore, numerous studies have assessed the effects of lesioning cholinergic nuclei with the selective cholinergic toxin 192 IgG saporin. Specific lesions of the basal cholinergic forebrain produced deficits in spatial memory tasks, including the Morris Water Maze (Berger-Sweeney et al., 2001; Frick et al., 2004; Nilsson et al., 1992), attentional and executive function tasks such as signal detection tasks and 5-choice serial reaction time (Burk and Sarter, 2001; McGaughy et al., 2002). Studies where mAChR subtypes are selectively knocked out, have further investigated the importance of different receptor subtypes. Of particular importance to this thesis, M_1 mAChR

knockout (KO) mice display performance deficits in tasks requiring PFC function; for example, M_1 mAChR KO display performance deficits in the win-shift radial arm maze learning task, however no deficits in hippocampal learning tasks such as the Morris Water Maze (Anagnostaras et al., 2003). In two touchscreen tasks, M_1 mAChR KO mice displayed impaired cognition with abnormal responding on a 5-choice serial reaction time task (Bartko et al., 2011) and delayed acquisition of a pairwise discrimination task (Gould et al., 2015). In contrast, M_4 mAChR KO mice display compulsive responding in the 5-choice serial reaction time task (Justinussen et al., 2020) and increased anxiolytic phenotypes, however normal long-term memory performance (Degroot and Nomikos, 2006).

1.1.4. Central Cholinergic Systems Role in Normal Sleep/Wake Architecture

The central cholinergic system has been reported to be important in maintaining normal sleep-wake architecture. Cholinergic neurons in the pontine brainstem and basal forebrain operate in conjunction with multiple neurotransmitter systems to activate the thalamocortical network and promote wakefulness (Luppi and Fort, 2019)(Figure 1.3). This explains the high levels of acetylcholine observed during active periods and low levels during inactive periods (Mitsushima et al., 1996). In addition, the cholinergic system has also been implicated in the modulation of rapid eye movement (REM) sleep, with studies showing that brainstem and forebrain cholinergic projections play a role in activating and maintaining REM sleep (Han et al., 2014; van Dort et al., 2015)(Figure 1.3). Interestingly there are relatively few studies with muscarinic receptor KO mice that display mAChR-dependent effects on sleep/wake architecture. One recent study showed that M_1 mAChR KOs displayed reduced non-REM (NREM) and REM sleep, with M_3 mAChR displaying reduced NREM sleep and M_1/M_3 double-KO mice having REM sleep almost completely eradicated (Niwa et al., 2018). Previous studies have shown no effect of M_2 or M_4 mAChR KO at baseline; however, following sleep deprivation, M_2 mAChR KO mice show no rebound in NREM sleep and a greater rebound in REM sleep (Turner et al., 2010). In support

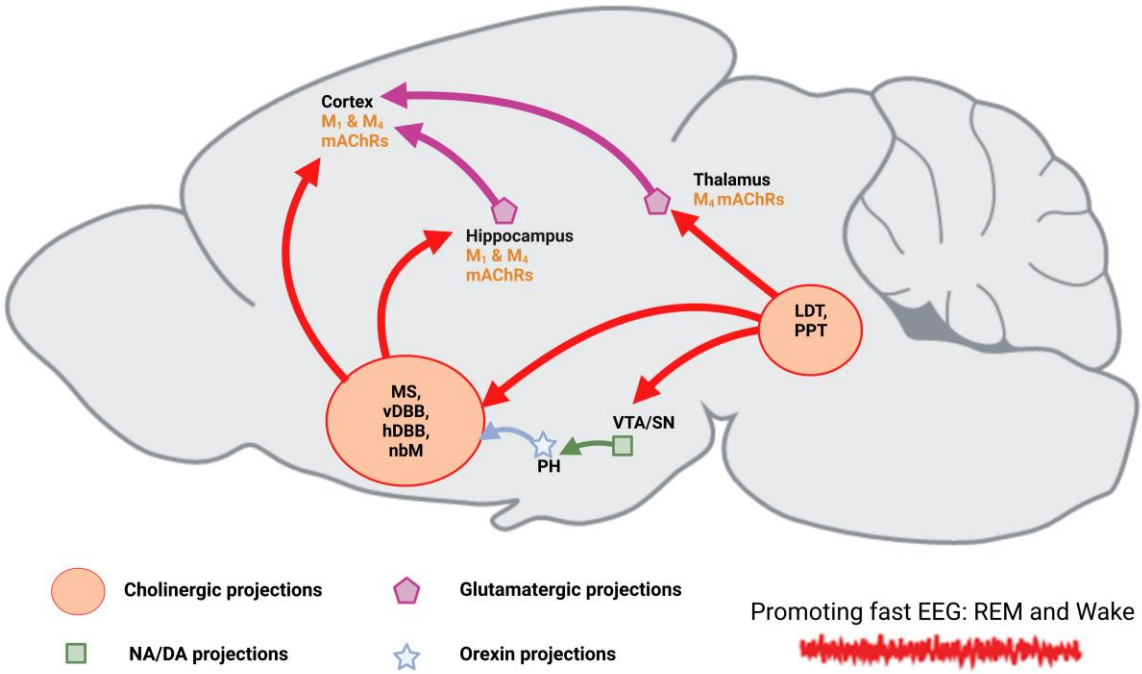

Figure 1.3. Cholinergic circuitry involved in modulating different arousal states. Red symbols indicate cholinergic neuronal pathways that are active during wake and/or REM sleep. Ascending pathways generate high-frequency oscillations during wake and/or REM sleep. Abbreviations: DA, dopamine; EEG, electroencephalogram; hDBB, horizontal limb of the diagonal band of Broca; LDT, laterodorsal tegmental nucleus; MS, medial septum; nbM, nucleus basalis of Meynert; PH, posterior hypothalamus; mAChR, muscarinic acetylcholine receptor; NA, noradrenaline; PPT, pedunculopontine tegmental nucleus; SN, substantia nigra; vDBB, vertical limb of the diagonal band of Broca; VTA, ventral tegmental area. Created in biorender.com

of this work, one study suggested that M_2/M_4 mAChR KO produced no change in REM sleep; however, M_3 mAChR KO reduced REM sleep (Goutagny et al., 2005).

In contrast, the cholinergic system is not believed to be involved in NREM sleep control. The networks responsible for modulating NREM sleep are thought to operate by reducing activity in the wake-promoting thalamocortical system largely through the GABAergic effects of the ventrolateral preoptic nucleus, the nucleus accumbens and reticular thalamic nucleus (Luppi and Fort, 2019)(Figure 1.3). Furthermore, studies have indicated that low levels of acetylcholine during NREM sleep are important for memory consolidation (Gais and Born, 2004; Inayat et al., 2020).

1.2. Central Cholinergic System Changes in Non-pathological Aging

1.2.1. Central Cholinergic Changes in Clinical Populations

The integrity of the central cholinergic system can be divided into structural measures (i.e., changes in fiber density) or functional measures (i.e., changes in ACh release). Numerous cholinergic neuronal markers, including AChE, ChT, ChAT, and vAChT, have been used to assess anatomical changes in central cholinergic structure in non-pathological aging and dementia. These markers can be assessed ex vivo using immunohistochemistry (IHC), enzyme activity assays, or in vivo using positron emission tomography (PET) ligands. Bartus and colleagues reviewed numerous changes in measures of cholinergic innervation seen in AD, including ex vivo decreased ChAT activity in cortical, striatal, and hippocampal areas, and compared these to more mixed findings from studies assessing non-pathologically aged clinical participants and preclinical species. Based on these comparisons, Bartus et al. suggested an emerging role for the central cholinergic system in geriatric memory dysfunction (Bartus et al., 1982). In more recent studies in non-pathologically aged populations, it has been shown that decreased in vivo acetylcholinesterase activity, as measured using positron emission tomography (PET) ligand [11C]N-methyl-4-piperidyl acetate, correlates with decreased cognitive performance, providing further evidence that the cholinergic system is important for cognitive performance in non-pathological aging (Richter et al., 2014). Another study utilizing a cholinergic PET ligand ([^{18}F]fluoroethoxybenzovesamicol ([^{18}F]FEOBV)) binding the vAChT has suggested that an array of cholinergic projections decline with increasing age, with reduced [^{18}F]FEOBV binding observed in numerous cortical areas, caudate nucleus, cingulum, insula, para-hippocampal and hippocampal regions, amygdala, thalamic areas and the cerebellum (Kanel et al., 2022).

1.2.2. Anatomical Central Cholinergic Changes in Preclinical Species

In preclinical species, changes in central cholinergic structure and function have been observed in non-pathological aging. Structurally, assessments have focused on the cholinergic basal forebrain circuitry, where observed changes have varied between studies. In one study ChAT positive cell density and size did not decline in rats up to 25 months of age; however, in female rats, there was a reduction in the nerve growth factor receptor (NGF) receptor (tyrosine Kinase A (TrkA)) mRNA in ChAT expressing neurons (Gibbs, 1998). NGF signaling through the TrkA receptor provides trophic support for basal forebrain cholinergic neurons (Hefti et al., 1989), suggesting a potential reduction in trophic support for the cholinergic system in aged female rats in the previous study, however, these findings did not extend to male rats (Gibbs, 1998). In contrast, a study assessing double labeled ChAT and NGF-receptor cell body number and size revealed reductions in cell body number in the medial septum and ventral diagonal band in 24-month-old rats with cognitive impairments, and in the medial septum, ventral diagonal band, striatum, and nucleus basalis of Meynert all 30-month-old rats, and reductions in cell body size in the medial septum and diagonal band in all 24-month-old rats and the medial septum, ventral diagonal band, striatum, and nucleus basalis of Meynert in all 30-month-old rats (Fischer et al., 1992). Studies assessing cholinergic boutons within the rat cortex have indicated that aging produces a reduction in cholinergic boutons along pyramidal neurons, which is particularly pronounced in layer V in rats 29-37 months of age (Casu et al., 2002). In more recent studies, the PET tracer [18F]-FEOBV uptake has been shown to be reduced in hippocampal regions in aged rats (18-month-old) (M. Parent et al., 2012). A recent study in 25-month-old mice demonstrated a loss of cholinergic fiber density, through ChAT immunohistochemistry, in the dorsal hippocampus and parietal cortex (Xie et al., 2019). These data suggest that cholinergic degeneration may be observed in both cell bodies and terminal regions depending on the age, species, and strain of rodent being assessed.

In preclinical rodent studies, detailed functional assessments of the central cholinergic system, which have not been possible in clinical populations. In early Parkinson's disease it has been suggested that dopamine turnover increases to compensate for loss of dopaminergic neurons (C. S. Lee et al., 2000; Sossi et al., 2002), to date similar compensatory changes have not been observed in the cholinergic system. Microdialysis studies in 2-, 9- and 18-month-old rats revealed a 35-53% reduction in extracellular acetylcholine at 18 months (C. F. Wu et al., 1988). While a study assessing circadian change in extracellular acetylcholine suggested that aged rats (23-24-month-old) lose the normal circadian fluctuations in acetylcholine, whereas young rats (3-4-month-old) displayed increased extracellular acetylcholine during the active (dark) phase when compared to the inactive (light) phase (Mitsushima et al., 1996). In addition, a PET image study in non-human primates using [(18)F] (+)-4-fluorobenzyltrozamicol ((+)-[(18)F]FBT), targeting the vAChT, displayed an age-dependent reduction in uptake in the basal ganglia, although significant individual variability was noted (Voytko et al., 2001).

1.3. Sleep-Wake Architecture Disturbances and Changes in Cognition and Arousal in Non-pathological aging

1.3.1. Age-related Changes in Sleep/Wake Architecture in Clinical Populations

Numerous sleep disturbances develop during non-pathological aging, with various macro-sleep architecture parameters changing through adulthood before stabilizing at around 60 years old (J. Li et al., 2018). Specifically, total sleep time (TST), sleep efficiency (as defined by total sleep time divided by time in bed), percent of rapid eye movement (REM) sleep, and percent deeper slow wave NREM sleep decrease from young adults to old age (>60 years old), and stage 1 and stage 2 NREM sleep, and wake after sleep onset (WASO) increase (Ohayon et al., 2004). Of these changes, only sleep efficiency continues to decline into later life (Ohayon et al., 2004).

Studies assessing microarchitecture changes during aging have focused on changes in slow wave activity (SWA, 0.5-4Hz; delta frequencies) and changes in sleep spindles (12-16Hz, sigma frequencies) during NREM sleep, both of which are seen to decline from middle age (Carrier et al., 2001; Darchia et al., 2007). Studies have suggested that both delta power (SWA) (Kirov et al., 2009) and sleep spindles (Schabus et al., 2004) are important in memory consolidation and that decreases in these may play a role in age-related reductions in memory consolidation (Fogel et al., 2012).

1.3.2. Age-related Changes in Arousal and Cognition in Clinical Populations

Numerous age-related changes in cognition have been identified, which have been linked to deficits in cholinergic function. Drachmann and colleagues demonstrated that aged populations displayed impairments in both memory and non-memory-dependent cognitive tasks, mirroring the effects of the muscarinic antagonist scopolamine (Drachman and Leavitt, 1974). Further studies have assessed age-related cognitive changes, identifying reductions in speed of processing and long- and working-memory function (Park and Reuter-Lorenz, 2009). The scaffolding theory of aging and cognition (STAC) hypothesizes that compensatory activity in alternate brain areas, such as the prefrontal cortex, supports these declining cognitive functions (Park and Reuter-Lorenz, 2009). Supporting this hypothesis, functional imaging studies have suggested task-related overactivation of prefrontal cortical areas in normally aging participants (Cabeza et al., 1997; Reuter-Lorenz et al., 2000). Newhouse and Dumas hypothesized that this increased frontal cortical activation is due to a decline in circuits controlling the focus of attention, resulting in increased compensatory basal forebrain cholinergic activation in the frontal cortex. However, as the basal forebrain cholinergic system declines, this compensation can no longer occur, and cognitive deficits become apparent (Dumas and Newhouse, 2011).

Appropriate levels of arousal have been indicated to be vitally important in cognitive functioning (Yerkes and Dodson, 1908). Arousal can be measured through numerous EEG

methods; two of the more common methods are by using resting-state measures, where participants either sit quietly with eye's open or closed and spectral power is assessed, or through measuring EEG coherence, a measure of functional connectivity through synchronicity based on the phase of EEG signals (Srinivasan et al., 2007). Specifically, alpha and beta powers are commonly associated with increased wakefulness and arousal, with lower-frequency powers such as delta being sleep-promoting. Regarding coherence, increased alpha coherence in intrafrontocortical and fronto-occipitocortical leads have been associated with increased arousal (Cantero et al., 1999). In non-pathological aging, there are observed to be decreases in resting state lower absolute powers, delta, theta, and alpha, and increases in higher absolute powers, beta and gamma (Anderson and Perone, 2018; Jabès et al., 2021; Meghdadi et al., 2021). After 60 years of age, coherence in delta, theta, and alpha bands is seen to decrease (Meghdadi et al., 2021). These EEG changes differ significantly from the pattern seen in mild cognitive impairment and Alzheimer's Disease, which will be discussed in more detail below.

1.3.3. Age-related Changes in Sleep/Wake Architecture in Preclinical Species

Several recent studies have assessed age-related sleep changes in non-pathologically aged mice. Results have varied, with reductions in wakefulness and increases in NREM sleep observed during the active phase (McKillop et al., 2018; Panagiotou et al., 2017) and no significant change in REM sleep (McKillop et al., 2018) or reduced REM sleep during the inactive phase (Panagiotou et al., 2017) at 18-24 months of age. Delta power (SWA) increases following sleep deprivation has been shown to be attenuated in aged animals (McKillop et al., 2018); however, absolute spectral power from 2-7Hz (Panagiotou et al., 2017) or 2-10Hz (McKillop et al., 2018) during NREM sleep and overall NREM sleep delta power (SWA) is increased in aged mice (Panagiotou et al., 2017).

1.3.4. Preclinical Age-related Changes in Arousal and Cognition

Numerous studies have found impairment in several cognitive domains in non-pathologically aged mice when compared to young mice, with studies demonstrating robust age-related change from 17 months of age (Buscher et al., 2017). Studies using radial arm water maze and T-maze have revealed deficits in working and spatial memory (Krukowski et al., 2020; von Bohlen Und Halbach et al., 2006). Non-pathologically aged mice have also been demonstrated to have deficits in the Morris Water Maze task suggesting age-related deficits in spatial long-term and working memory (von Bohlen Und Halbach et al., 2006). On the novel object recognition task, non-pathologically aged mice have been seen to have reduced recognition following both short delays (3-minute) (Soontornniyomkij et al., 2012) and longer delays (24-hours) (Fahlström et al., 2011). On more complex touchscreen tasks, non-pathologically aged mice have been demonstrated to have marked deficits in acquiring pairwise discrimination and greater errors on an automated search task (Buscher et al., 2017). Similar to mice, non-pathologically aged rats display deficits in numerous memory tasks when compared to young rats (Hamezah et al., 2017; Lomidze et al., 2021).

Age-related changes in arousal, as quantified by changes in qEEG, have been less well characterized in preclinical species. One study assessed qEEG changes during rest and active periods in aging compared to young and middle-aged cohorts and described that similar to clinical AD populations, aged mice (20-24-month-old) display an increase in delta power (1-2Hz) and a maximal peak at lower frequencies (6-8Hz vs. 8-10Hz) during active recording (del Percio et al., 2017). It is important to note that this study only assessed frequencies up to 30Hz.

1.4. Central Cholinergic System Degeneration in Alzheimer's Disease and Associated Change in Sleep/Wake Architecture, Arousal, and Cognition

Some of the first evidence for central cholinergic degeneration in Alzheimer's Disease (AD) was from a small study demonstrating that patients with senile dementia of Alzheimer's type

had significant reductions of choline acetyltransferase (ChAT) activity in the amygdala, cortex, and hippocampal areas (Davies and Maloney, 1976). Following this, a case study indicated an individual with senile dementia of Alzheimer's type displayed markedly reduced nissl staining of neuronal cells in the cholinergic nucleus basalis of Meynert compared to an aged-matched control (Whitehouse et al., 1981). These findings were later extended to a broader population of individuals who had died from senile dementia of Alzheimer's type (Whitehouse, Price, Struble, Clark, Coyle, and DeLong, 1982).

In AD patients, a decrease in TrkA and an increase in NGF are observed. It is hypothesized that this disruption in NGF support for the basal forebrain cholinergic system underlies the loss of central cholinergic integrity seen in AD and aging (Mufson et al., 1999). Assessment of cortical ChAT immunohistochemical (IHC) staining in mild cognitively impaired individuals and patients with early AD has revealed no decline in cortical ChAT-positive fibers or varicosities in MCI; however, marked decreases in early AD (Ikonomovic et al., 2007). This contrasts with cortical ChAT activity, which remains constant through early AD, only declining in more severe AD (Davis et al., 1999; Tiraboschi et al., 2000).

Since the advancement of PET and magnetic resonance imaging (MRI) modalities, an increasing number of studies have assessed central cholinergic integrity during the progression of AD. These studies have focused on PET tracers targeting cholinergic markers such as the vesicular acetylcholine transporter (vAChT) or MRI studies assessing the volume of the cholinergic forebrain. Reduction in basal forebrain cholinergic volume has been indicated as a reliable predictor of entorhinal and neocortical neurodegeneration and constitutes an early event in the development of AD (Fernández-Cabello et al., 2020). Additionally, patients with subjective cognitive decline (SCD), a risk factor for the development of preclinical AD, have been suggested to have decreased basal forebrain cholinergic volume as measured by MRI (Scheef et al., 2019). PET studies utilizing [18F]-fluoroethoxybenzovesamicol ([18F]-FEOBV) (targeting the vAChT) have shown that patients with AD have reduced uptake in numerous cortical areas, which

correlated with cognitive performance, suggesting [18F]-FEOBV PET imaging may represent a sensitive biomarker for AD (Aghourian et al., 2017). More recent studies have extended this work, suggesting that cortical [18F]-FEOBV uptake is reduced in patients with mild cognitive impairment (MCI), and in the absence of cortical atrophy, this reduction in [18F]-FEOBV uptake correlated with cognitive performance, suggesting [18F]-FEOBV imaging is a more sensitive biomarker of central cholinergic structure than general cortical atrophy loss, and that cholinergic degeneration is an important factor in the loss of cognitive function in early dementia (Xia et al., 2022). Furthermore, recent work with the PET tracer [11C]MK-6884 have displayed reduced sensitivity to donepezil treatment in AD populations, suggesting a reduction in cholinergic tone in AD (W. Li et al., 2022).

In clinical AD, sleep disturbances are a commonly observed symptom and often occur prior to cognitive symptoms and AD diagnosis (F. Zhang et al., 2019). Numerous studies have identified sleep disturbances as a risk factor for the future development of AD (Benedict et al., 2015; Lim et al., 2013). These sleep disturbances are suggested to have a bi-directional relationship with AD pathology, whereby increased AD pathology leads to sleep disturbances, and sleep disturbances lead to increases in AD pathology (Bubu et al., 2017; Shokri-Kojori et al., 2018; Yulug et al., 2017). Clinical studies have identified that reduced slow wave activity during NREM sleep predicts increased cerebrospinal fluid β-amyloid (Ju et al., 2017) and, more specifically, decreases in lower delta frequencies and increases in higher delta frequencies correlate with future β-amyloid beta accumulation (Winer et al., 2020).

A wide array of cognitive deficits is observed in AD. MCI is often observed as a prodrome to AD and may be either amnestic or non-amnestic, with amnestic being the more common form (Sanford, 2017). To be diagnosed with MCI, a patient must present with both subjective and objective cognitive decline. From here, a patient may progress to AD (Sanford, 2017), where they may develop an array of cognitive symptoms, including memory decline, depressed mood, personality changes, behavioral disturbances, language difficulties, disorientation, and psychosis

(Bature et al., 2017). Changes in qEEG arousal measures of arousal in MCI and AD have been well characterized; patients with MCI and AD display a shift to lower frequency powers typified by increases in delta and theta and reductions in alpha power resulting in an increased theta to alpha ratio (Meghdadi et al., 2021).

1.5. Cholinergic Therapeutic Strategies for the Treatment of Alzheimer's Disease and their Effects on Sleep-Wake Architecture and Cognition

1.5.1. Acetylcholinesterase Inhibitors

To date, acetylcholinesterase inhibitors (AChEIs) represent the only FDA-approved treatment for the cognitive impairments associated with AD that specifically target the cholinergic system. AChEIs block the degradation of ACh by AChE, resulting in increased synaptic levels of ACh (Sharma, 2019). While AChEIs produce modest therapeutic effects on cognitive impairments during the early stages of AD, these drugs are associated with dose-limiting adverse effects due to nonselective activation of central and peripheral muscarinic acetylcholine receptors (mAChRs) (Galimberti and Scarpini, 2016). Donepezil displays excellent selectivity for the rat (rAChE) over the rat butyrylcholinesterase (rBuChE), with a 50% maximal inhibitory concentration (IC_{50}) of 5.7nM at rAChE and 7138nM at rBuChE. Donepezil reaches a maximal concentration (T_{max}) 30-60 minutes after dosing following oral administration (per os, P.O.) and has a half-life ($t_{1/2}$) of over 6 hours. For donepezil, this in vivo data was derived from functional measures of AChE activity at discrete time points following dosing rather than concentration measures (Sugimoto et al., 1995). Additionally, donepezil shows excellent brain penetration with a total brain-to-plasma ratio (K_p) of 6.1-8.4 in rats (Kosasa et al., 2000) (See Figure 1.4)

Effects of AChEIs on sleep-wake architecture in clinical populations are mixed; one study describes no effect on REM sleep, but increased stage 2 NREM sleep following donepezil

treatment in patients with AD (Cooke et al., 2006), while others describe increases in REM sleep, which correlated with cognitive improvement (dos Santos Moraes et al., 2006). Furthermore, it is apparent that the time of dosing is important, with morning dosing of AChEIs subjectively improving sleep quality and reducing daytime drowsiness compared to evening dosing (Song et al., 2013). Studies with cholinergic agonists, such as RS86, have shown a shortened REM sleep latency, increased REM sleep, and decreased NREM sleep (Riemann et al., 1988), consistent with the importance of low cholinergic activation during NREM sleep and the role of cholinergic signaling in REM sleep.

1.5.2. Xanomeline (In phase III trials)

Early clinical studies with xanomeline, an M_1/M_4-mAChR subtype-preferring orthosteric agonist, showed significant efficacy in treating AD-related behavioral disturbances and trends toward improving reaction time and verbal memory deficits (Bodick et al., 1997; Veroff et al., 1998). Xanomeline and other M_1-preferring orthosteric agonists also produced pro-cognitive effects in rodents and NHPs (C. K. Jones et al., 2012), yet have failed in clinical development due to off-target activation of peripheral mAChRs similar to those observed with AChEIs (Bodick et al., 1997). In rats, xanomeline displays modest selectivity for the M_1 and M_4 mAChRs with a 50% maximally efficacious concentration (EC_{50}) of 0.3 and 0.1 μM at the rat M_1 (rM_1) and rat M_4 (rM_4) mAChRs respectively. At the rat M_2 (rM_2), rat M_3 (rM_3), and rat M_5 (rM_5) mAChRs xanomeline displays EC_{50}'s of 3, 1, and 10 μM respectively, highlighting the lack of selectivity. Xanomeline in rats displayed a P.O. T_{max} of 0.75hr with a $t_{1/2}$ of 0.54 hours, making it ideally suited for in vivo rodent studies (Bymaster et al., 1997)(See Figure 1.4). Currently, xanomeline compounded with the peripherally restricted muscarinic antagonist trospium (KarXT) is in clinical trials for the treatment of schizophrenia (Brannan et al., 2021) and psychosis in AD (ClinicalTrials.gov: NCT05511363). Clinical sleep studies with xanomeline are lacking; however, in young rats,

xanomeline increased wakefulness, gamma power during wake, and reduced delta power (SWA) during NREM sleep (Gould et al., 2016).

1.5.3 Targeting the Cholinergic System Through Allosteric Modulation

Of the five different mAChR subtypes activated by ACh (M_1-M_5), the M_1 mAChR is highly expressed postsynaptically in brain regions that regulate arousal, sleep, and cognition, including the cortex, striatum, and hippocampus (Levey et al., 1991, 1995; Marino et al., 1998; Rouse et al., 1998, 1999). The M_4 mAChR is found postsynaptically on striatal D_1-expressing medium spiny neurons (Foster et al., 2016), presynaptically as inhibitory autoreceptors on cholinergic neurons (Tzavara et al., 2003), or as inhibitory heteroreceptors on corticostriatal glutamatergic neurons (Pancani et al., 2014). Both are expressed in hippocampal, cortical, striatal, and thalamic regions (Lebois et al., 2018; Levey, 1993), areas which are of particular interest for AD pathology (Sengoku, 2020) and sleep control (Gent et al., 2018). Both selective M_1 activation (Ghoshal et al., 2016; Gould et al., 2015; Lange et al., 2015; Moran et al., 2018; Rook et al., 2018) and selective M_4 activation (Bubser et al., 2014; Gould et al., 2018; Lange et al., 2021) have been seen to enhance cognitive performance in preclinical species. Thus, activation of M_1 and M_4-mAChRs are thought to be promising strategies for the symptomatic treatment of MCI and AD-related cognitive deficits. Using an alternative strategy for developing subtype-selective activators of M_1 and M_4-mAChRs, our group and others have focused on identifying ligands that target less highly conserved regions of the receptor, termed allosteric sites, which are distinct from the highly conserved ACh binding site. This approach has resulted in the discovery of multiple M_1 and M_4 mAChR positive allosteric modulators (PAMs), including VU0453595 (M_1) and VU0467154 (M_4), with greater than 30-fold selectivity for M_1 or M_4 respectively over the other mAChR subtypes and with suitable pharmacokinetic (pK) properties for dosing in rodent and non-human primate (NHP) species (Bubser et al., 2012, 2014; Conn, Lindsley, et al., 2009; Ghoshal et al., 2016; C. K. Jones

et al., 2012; Moran et al., 2018). VU0453595 and VU0467154 do not directly activate their respective receptors but potentiate the effects of presynaptically released ACh, thereby maintaining the spatial and temporal pattern of endogenous central acetylcholine signaling (C. K. Jones et al., 2012). The M_1 mAChR PAM, VU0453595, displays good potency at rM_1 and human M_1 (hM_1) mAChRs, 3.2 and 4.6 μM, respectively. VU0453595 displays good M_1 selectivity over rM_2-rM_5 with an EC_{50} greater than 30 μM at all receptors. Furthermore, VU0453595 displays characteristics ideally suited to in vivo studies with a mouse T_{max} of 0.25 hours following intraperitoneal (I.P.) dosing, a mouse $t_{1/2}$ of 0.56 hours, and excellent brain penetration in the mouse with a K_p of 0.13 and an unbound brain to unbound plasma ratio ($K_{p,uu}$) of 1.1. (Ghoshal et al., 2016) (Figure 1.4). VU0467154 displays excellent potency at the rM_4 with an EC_{50} of 17.7nM and a slightly reduced potency at the hM_4 with an EC_{50} of 627nM. VU0467154 displayed excellent selectivity over $rM_{1,2,3,5}$ with an EC_{50} of >30 μM at all receptors. VU0467154 displays pharmacokinetic properties conducive to in vivo studies with a mouse T_{max} of 0.5-1 hour and good brain penetration with a K_p/$K_{p,uu}$ of 0.64/0.41 in the mouse. The rat mouse $t_{1/2}$ is unpublished for VU0467154; however, the rat $t_{1/2}$ is 5.7 hours (Bubser et al., 2014). In addition to highly selective allosteric modulators, recent work by our group has identified novel compounds which are orthosteric agonists but display excellent selectivity for specific mAChRs subtypes. Particular to this dissertation, the M_4 mAChR antagonist VU6028418 displays excellent subtype selectivity with an IC_{50} of 4.1nM at hM_4 mAChR and 76nM at mouse M_4 (mM_4) mAChRs with the IC_{50} at $hM_{1, 2, 3, 5}$ >3 μM. In mice, VU6028418 displays a P.O. T_{max} of 6.7 hours and good penetration of the brain with a K_p of 2.3. Mouse $t_{1/2}$ was protracted and not calculated; however, rat $t_{1/2}$ was 13 hours (Spock et al., 2021)(Figure 1.4).

While both M_1 and M_4 mAChR PAMs have enhanced cognition in young animals, the efficacy of PAMs will vary depending on the available acetylcholine. As addressed earlier, central cholinergic integrity declines with aging, and cholinergic signaling varies depending on the circadian time point. Further, the effects of M_1 and M_4 activation on sleep/wake architecture and

EEG spectral power have not been fully characterized. In this dissertation, I will compare the sleep/wake architecture and qEEG effects of the direct-acting M_1/M_4-preferring orthosteric agonist xanomeline to the existing standard of care for AD, donepezil, in young and non-pathologically aged mice. Following this, I dissect the M_1 and M_4-dependent effects of xanomeline utilizing the M_1 mAChR PAM VU0453595 and the M_4 mAChR PAM VU0467154 in young and aged animals across active and inactive phases of the circadian cycle, while correlating efficacy at different ages with central cholinergic structure. These data will provide crucial translational information when considering these compounds' utility and potential dosing times in disease populations with markedly reduced central cholinergic integrity.

CHAPTER 2

The M_1/M_4-Preferring Muscarinic Cholinergic Receptor Agonist Xanomeline Reverses Wake and Arousal Deficits in Non-pathologically Aged Mice

2.1. Introduction

Basal forebrain cholinergic degeneration has been identified as an important factor in the clinical symptomatology of Mild Cognitive Impairment (MCI) and Alzheimer's Disease (AD) (Bartus et al., 1982; Herholz, 2008; Peter et al., 2016; Terry and Buccafusco, 2003). Previous studies have demonstrated decreases in cortical and hippocampal cholinergic markers in aging and AD patient populations (Aghourian et al., 2017; Dumas and Newhouse, 2011; Kanel et al., 2022; Whitehouse et al., 1981; Whitehouse, Price, Struble, Clark, Coyle, and DeLong, 1982). In addition, preclinical and clinical studies have reported that reductions in cholinergic markers correlate with deficits in cognitive performance (Richter et al., 2014; F. Wang et al., 2009; Xia et al., 2022). Moreover, the cholinergic system has been shown to be crucial in modulating normal sleep/wake architecture and arousal with basal forebrain and brainstem cholinergic projections regulating wakefulness and rapid eye movement (REM) sleep (Han et al., 2014; van Dort et al., 2015; Y. Wu et al., 2020; Xu et al., 2015). Given the importance of the cholinergic system in sleep/wake architecture control, age- and AD-related cholinergic degeneration have also been implicated in sleep/wake architecture and arousal deficits (Grossberg, 2017; Montplaisir et al., 1998; Wisor et al., 2005).

Boosting cortical cholinergic signaling through indirect-acting muscarinic cholinergic receptor agonists such as acetylcholinesterase inhibitors (AChEIs) is the primary treatment for cognitive impairments in AD (Breijyeh et al., 2020). The mechanism of action for AChEIs is through the prevention of the breakdown of synaptic acetylcholine (ACh) (Sharma, 2019).

However, AChEIs produce only modest clinical efficacy due to progressive AD-related reductions in basal forebrain cholinergic synaptic signaling (Rogers et al., 1998; Winblad et al., 2001). AChEIs are also associated with numerous dose-limiting side effects, including nausea and diarrhea resulting from non-selective activation of peripheral muscarinic acetylcholine receptors (mAChRs) (Dunn et al., 2000; Galimberti and Scarpini, 2016). Of the five different mAChR subtypes that are activated by ACh (M_1-M_5), M_1 and M_4 are highly expressed in cortical and limbic regions thought to be associated with arousal and cognition (Levey, 1993; Levey et al., 1995; Marino et al., 1998), whereas M_2 and M_3 display central and peripheral expression and are linked to the peripherally mediated adverse effects of AChEIs (Felder et al., 2018).

As an alternative to AChEIs, multiple studies have investigated the effects of direct-acting muscarinic cholinergic receptor agonists that target the M_1 and/or M_4 mAChR subtypes for the treatment of impaired arousal and cognition in MCI and AD (Bodick et al., 1997; Hollander et al., 1987; Veroff et al., 1998). For example, in one large multicenter trial, the M_1/M_4-preferring muscarinic cholinergic receptor agonist xanomeline produced significant effects on the behavioral disturbances in AD with a trend towards improvement in cognition (Bodick et al., 1997; Veroff et al., 1998). However, xanomeline, similar to other direct-acting muscarinic cholinergic receptor agonists, failed during clinical development due to dose-limiting adverse effects from the activation of peripheral mAChR subtypes (Bender et al., 2017). Recent clinical studies indicate that formulation of xanomeline with the peripherally restricted non-selective muscarinic receptor antagonist trospium, known as KarXT, may provide a broader therapeutic index for the use of direct-acting muscarinic cholinergic agonists (Brannan et al., 2021) (ClinicalTrials.gov: NCT03697252, NCT04659161, NCT05511363).

While accumulating evidence supports further clinical development of direct-acting muscarinic cholinergic receptor agonists, there have been limited studies to evaluate the effects of this mechanism on disruptions in sleep-wake architecture and/or arousal in non-pathological aging, MCI, and AD patient populations. To date, previous electroencephalography (EEG) studies

by our group and others have investigated the effects of indirect- and direct-acting muscarinic cholinergic receptor agonists on the promotion of cholinergic signaling and subsequent changes in sleep/wake architecture and arousal during the inactive phase of young rodents (Amat-Foraster et al., 2017; Gould et al., 2016, 2020; Montani et al., 2021), when basal ACh levels are low (Mitsushima et al., 1996). When dosed in young rats during the inactive phase, both the AChEI donepezil and xanomeline promoted wakefulness and/or increased gamma power during wake (Amat-Foraster et al., 2017; Gould et al., 2016, 2020; Montani et al., 2021), a well-characterized correlate of arousal (Buzsáki and Silva, 2012). Consistent with these findings, AChEIs have been reported to boost arousal in AD patients, as shown by a significant shift from low to high spectral power (Balkan et al., 2003). However, AChEIs have also been shown to produce robust sleep disruptions in individuals with AD (Dunn et al., 2000; Ridha et al., 2018). In a recent meta-analysis of clinical studies in both healthy and AD patient populations, donepezil reduced stage 2 non-REM (NREM) sleep, sleep efficiency, and total sleep time (Hsieh et al., 2022). We have also demonstrated in young rats that donepezil dose-dependently decreases delta power (SWA; slow wave activity) during NREM sleep, a recognized measure of NREM sleep quality (Gould et al., 2020). Studies with the direct-acting muscarinic cholinergic agonist RS-86 revealed reductions in NREM sleep duration in healthy participants (Nissen, Power, et al., 2006), while xanomeline decreased NREM sleep duration and delta power (SWA) during NREM sleep in young rats when dosed in the inactive phase (Gould et al., 2016). Yet, despite these studies in young preclinical species and healthy volunteers, little is known about the effects of direct-acting muscarinic cholinergic agonists on arousal and sleep-wake architecture in non-pathologically aged rodents or clinical populations across the circadian cycle.

Given the changes in ACh signaling across the circadian cycle (i.e., high in the active phase and low in the inactive phase (Mitsushima et al., 1996)) and with aging (i.e., age-related reductions (Mitsushima et al., 1996)), the current studies provide the first systematic assessment of the effects of xanomeline, in comparison with donepezil, on sleep/wake architecture, arousal,

and sleep quality in aged and young mice across the circadian rhythm. We observed that aged mice displayed pronounced wake fragmentation and reductions in arousal during the active phase that could be reversed by xanomeline but not donepezil when dosed in the active phase. These studies provide the foundation for future sleep/wake architecture and spectral power assessments with xanomeline and other direct-acting muscarinic cholinergic receptor agonists in disease models of AD.

2.2 Methods

Subjects

Young adult (3-4-month-old, n=28) and aged (19-20-month-old, n=40; 28 for EEG, 12 for assessment of cholinergic side effects) wildtype male C56BL/6J mice (The Jackson Laboratory) served as subjects. Prior to study initiation, all mice were socially housed. Following surgical implantation of EEG telemetry devices, all animals were individually housed. Mice were housed in humidity-controlled rooms and maintained in a 12/12hr light-dark cycle with food and water available *ad libitum*. All studies 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*.

Compounds

Xanomeline (synthesized in-house, 3-30 mg/kg) and donepezil (AstaTech inc, Bristol, PA, 0.1-3 mg/kg) were dissolved in saline. All compounds were dosed at a volume of 10 ml/kg via intraperitoneal (I.P.) injection. The dose range for both has been shown to modulate sleep/wake architecture in rats(Gould et al., 2016, 2020), and the top dose of both was where dose-limiting adverse side effects were observed (Table 2.5 and 2.6).

Electroencephalography

Surgery

A telemetric transmitter (HD-X02, Data Science International [DSI], Minneapolis, MN) was implanted in all mice using previously described methods(Fisher et al., 2020; Gould et al., 2020). A 2-3cm midline incision was made over the skull. A frontoparietal EEG lead was placed, with the frontal co-ordinate at +1.5mm AP, -2mm ML and the parietal co-ordinate at -3mm AP, 2mm ML, which was secured with screws and covered with dental cement (Patterson Dental, Saint Paul, MN). A second biopotential lead for recording the electromyogram (EMG) was placed in the nuchal muscle. Mice were recovered for a minimum of 10-days post-surgery prior to recording.

EEG recording and sleep staging

EEG and EMG recordings were performed for 24-hours starting at either lights on (inactive phase) or lights off (active phase) with the mice housed in their home cage. Ponemah software (v3.0, DSI) was used to capture EEG and EMG waveforms. A wireless receiver (RCP-1, DSI) below each home cage transmitted data which was continuously sampled at 500Hz. Dosing with xanomeline (3-30 mg/kg or saline vehicle i.p.) or donepezil (0.1-3 mg/kg or saline vehicle i.p.) was performed 2-3-hours in to either the active or inactive phase. For all experiments time is displayed in zeitgeber time, where ZT 0 indicates transition from the active to the inactive phase (lights off to lights on).

Following recording, all traces were manually scored by trained observers blinded to age and dose. The recordings were scored in 5-second epochs using Neuroscore 3.3.1 software (DSI) as wake, NREM sleep, or REM sleep based on previously published characteristic patterns by our group(Fisher et al., 2020; Gould et al., 2016, 2020; Nedelcovych et al., 2015). The duration of time in each state (wake, NREM sleep, and REM sleep), separated into 2-hour, or 24-hour bins, served as the primary dependent measures to assess age and pharmacological effects. Fragmentation of sleep and wake was assessed by calculating average NREM sleep or wake bout length and number of NREM sleep or wake bouts for the 8 hours following dosing, thus remaining within the phase of dosing.

qEEG spectral power analysis

Once the data was divided by sleep stage relative spectral power from the quantitative EEG (qEEG) trace was calculated in 1Hz bins between 0.5 and 80Hz using a Fast Fourier Transformation (FFT) with a Hamming window overlap ratio of 0.5. Within each 1Hz interval, relative spectral power was binned by sleep stage (wake, NREM sleep or REM sleep). To understand pharmacological effects this was averaged across a pre-dose baseline, 1-2-hours following light change and a post-dose period, 1-2-hours following dosing. The post-dosing period is then represented within wake, NREM sleep and REM sleep respectively as a percent change to the predose period in the same state. Power band analysis across time within wake and NREM sleep were calculated by binning the spectral power from 0.5-4Hz (delta), 4-8Hz (theta), 8-13Hz (alpha), 13-30Hz (beta), and 30-80Hz (gamma) during wake and NREM sleep respectively. This was averaged each hour from 2-hr predose to 8-hr post-dose and represented relative to the 1-2-hours following light change. When assessing age-dependent changes, the 1-hr periods are normalized to the same 1-hr periods in young mice.

Assessing cholinergic adverse effects

Donepezil and xanomeline effects on autonomic and somatomotor function were assessed in non-pathologically aged C57BL/6J mice in the active and inactive phases. Assessments were performed 30, 60, 120, and 240 min after i.p. administration of 30 mg/kg xanomeline, 3 mg/kg donepezil or saline vehicle. For assessment, a modified Irwin neurological test battery (Irwin, 1968) was used as described in our previous work (Bubser et al., 2014). In brief, numerous autonomic and somatomotor behavioral endpoints were observed by blinded, trained observers (see Tables 2.5 and 2.6 for a complete list of behaviors assessed), and each behavior was scored as 0 (normal), 1 (mild effect) or 2 (marked effect). For each behavioral endpoint, the score was averaged across subjects and then the sum of the average scores for all the behavioral endpoints was used to calculate the total score.

Statistics

qEEG analysis and sleep/wake architecture are displayed as means ± S.E.M. Two-way repeated analysis of variance (repeating by both factors for pharmacological studies, repeated by one factor for age-related comparisons) were used when assessing relative spectral power change from 0.5-80Hz, individual power bands across time and for all sleep/wake architecture assessments. When data was absent due to animals not entering into NREM or REM sleep during the analysis period a repeated measures mixed effects model (REML) was applied. If data for an entire dose was absent at a given time point when assessing individual power bands across time, two-way repeated analysis of variance (or REMLs) were applied, one from the start of recording until the highest dose group displayed NREM sleep (including vehicle and all doses except the top dose), and one from the initiation of NREM sleep in the highest dose group following dosing (including vehicle and all doses). When assessing wake and NREM sleep average bout length and bout number an unpaired t-test was used to compare young and aged mice, and a repeated measures one-way analysis of variance (or REML if missing data due to mice not entering NREM sleep) to compare dosing conditions. A Sidak's multiple comparison test was performed to compare young and aged cohorts. Otherwise, a Dunnett's multiple comparison test was performed to compare dosing conditions to vehicle. For the modified Irwin neurological test battery, a total adverse event score was compared using a two-way analysis of variants with the vehicle condition grouped within phase, as a saline vehicle was used for both compounds and no differences were seen between vehicle treatments. A Tukey's multiple comparison test was used to compare main effect of dose across vehicle, xanomeline and donepezil treated conditions within phase. All statistical analysis and graphing were performed using GraphPad Prism version 9.4.1 (see Table 2.4 for full statistical analysis).

2.3. Results

Non-pathologically aged mice displayed fragmentation of wakefulness and decreased REM sleep during the active phase and decreased NREM sleep in the inactive phase.

To understand the changes in sleep-wake architecture associated with non-pathologic aging we compared the baseline sleep-wake architecture and spectral power characteristics during saline vehicle treatment in young (3-4-month-old) and aged (19-20-month-old) C57B6/J mice from both the xanomeline and donepezil dosing studies. During the active (lights off) phase we observed no change in wake duration on *posthoc* analyses (2-hour bins: age, p=0.0571; time, p<0.0001; age x time, p=0.0008 (Figure 2.1A) and 12-hour bins: age, p=0.0571; time, p<0.0001; age x time, p=0.0771 (Figure 2.1D)), an age-related increase in NREM sleep at ZT 16 (Zeitgeber time, where ZT 0 is transition from lights off to on) (age, p=0.1100; time, p<0.0001; age x time p=0.0001) (Figure 2.1B) with no change in total NREM (age, p=0.1100; time, p<0.0001; age x

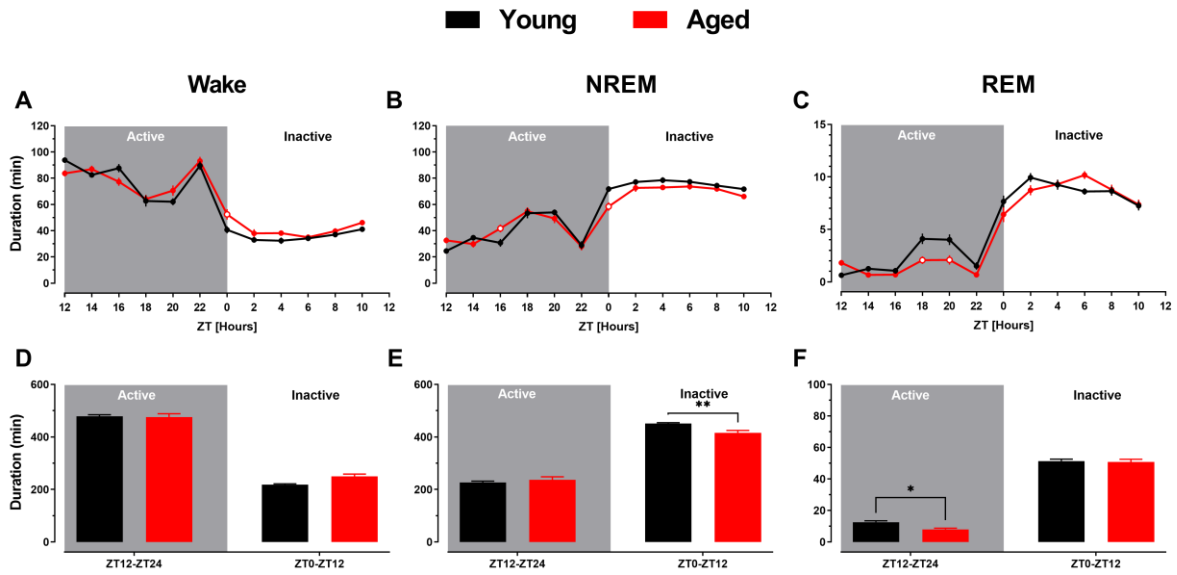

Figure 2.1. **Non-pathologically aged mice displayed reduced NREM sleep during the inactive phase and reduced REM sleep during the active phase.** Shown is the duration of time in wake (A, D), NREM sleep (B, E) and REM sleep (C, F) in young (3-4-month-old) and non-pathologically aged (19-20-month-old) mice. Compared to young mice, non-pathologically aged mice displayed significantly increased wake at ZT 0, with no overall change in either phase (A, D). Non-pathologically aged mice displayed significantly increased NREM sleep at ZT 16 and significantly decreased NREM sleep at ZT 0, with a significantly decreased total NREM sleep during the inactive phase (B, E). Non-pathologically aged mice displayed a significantly decreased REM sleep at ZT 18 and 20 (C) and a significantly decreased total REM sleep between ZT 12-ZT 24 (F). Data are expressed as means ± S.E.M. of 2-hour bins (A-C); total duration of time in minutes in wake, NREM sleep and REM sleep respectively ± S.E.M in 12hrs bins (D-F); n=28/group; open circles indicate p<0.05 (C), * indicates p<0.05, ** p<0.01, compared to young (repeated measures (RM) 2-way ANOVA matching by time followed by Sidak's test). See table 2.4 for full statistical analysis.

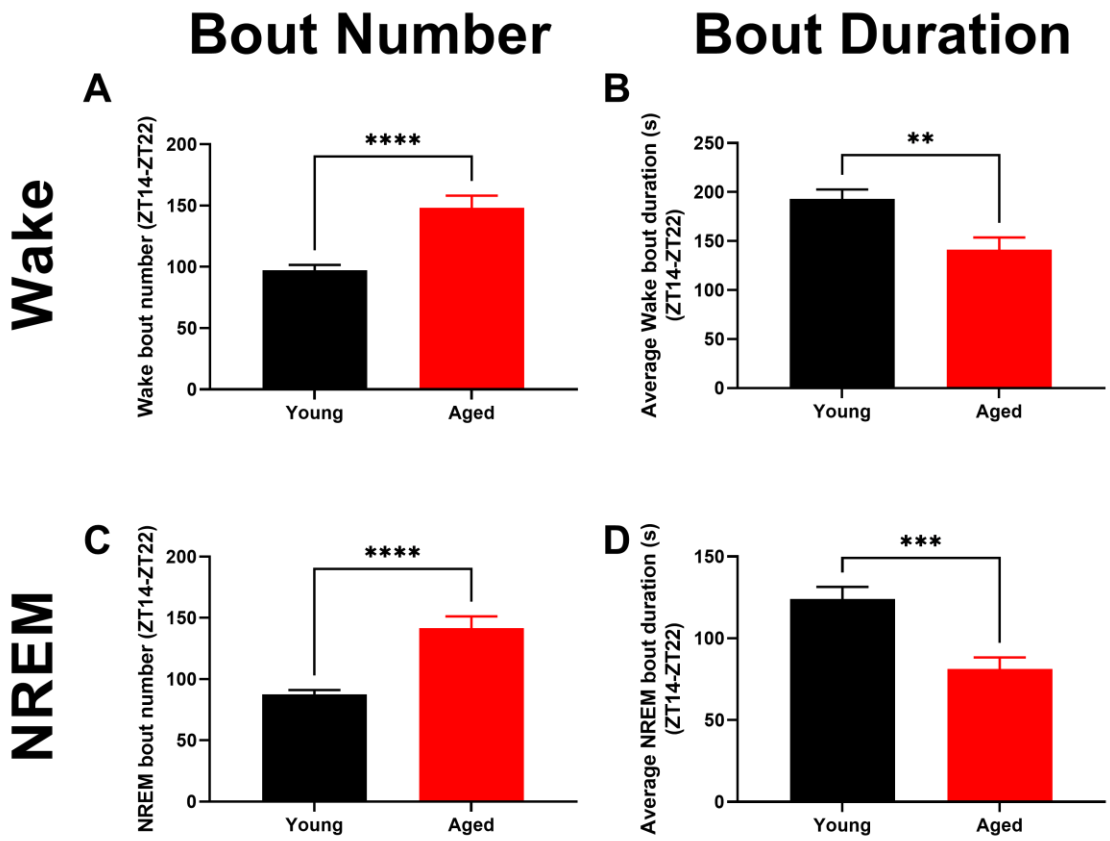

Figure 2.2. Non-pathologically aged mice displayed fragmented wakefulness during the active phase. Shown is the wake bout number (A), wake bout duration (B), NREM sleep bout number (C) and NREM sleep bout duration (D) in young and non-pathologically aged mice. Non-pathologically aged mice displayed increased wake bout number (A), reduced wake bout duration (B), increased NREM sleep bout number (C) and reduced NREM bout duration (D). Data are expressed as overall means ± S.E.M., n=28/group. ** indicates p<0.01, *** p<0.001 and **** p<0.0001 compared to young (unpaired t-test). See table 2.4 for full statistical analysis.

time, p=0.0107) (Figure 2.1E), and an age-related decrease in REM sleep at ZT 18 and 20 (age, p=0.0260; time and age x time, p<0.0001) (Figure 2.1C) with an overall decrease in REM sleep observed (age, p=0.0260; time p<0.0001; age x time, p=0.1397) (Figure 2.1F). When assessing fragmentation of wakefulness during the active phase, we observed that non-pathologically aged mice displayed increased wake bout number (p<0.0001) (Figure 2.2A) and decreased wake bout duration (p<0.0017) (Figure 2.2B), and consequently displayed increased NREM sleep bout number (p<0.0001) (Figure 2.2C) and decreased NREM sleep bout duration (p=0.0001) (Figure

Table 2.1. Non-pathologically aged mice displayed no change in wake or NREM sleep fragmentation during the inactive phase

	Wake		NREM	
	Bout number, ZT2-10 (SEM)	**Average bout duration, ZT2-10 (s), (SEM)**	**Bout number, ZT2-10 (SEM)**	**Average bout duration, ZT2-10 (s) (SEM)**
Young	103.9 (2.668)	72.09 (2.745)	103.7 (2.735)	186.7 (5.755)
Aged	107.4 (3.380)	76.54 (14.01)	107.7 (3.321)	177.9 (6.234)
T-test	$T_{52} = 0.8171$	$T_{52} = 1.157$	$T_{52} = 0.9211$	$T_{52} = 1.037$
P value	0.4176	0.2526	0.3612	0.3044

2.2D). During the inactive (lights on) phase, we observed increased wake at ZT 0 (age, p=0.0571; time, p<0.0001; age x time, p=0.0008) (Figure 2.1A) with no overall change in wake (age, p=0.0571; time, p<0.0001; age x time, p=0.0771) (Figure 2.1D), a decrease in NREM sleep at ZT 0 (age, p=0.1100; time and age x time p=0.0001) (Figure 2.1B) with an overall decrease in NREM sleep (age, p=0.1100; time, p<0.0001; age x time, p=0.0107) (Figure 2.1E) and no change REM sleep (age, p=0.0260; time p<0.0001; age x time, p=0.1397) (Figure 2.1F). During the inactive phase no change in wake or NREM bout number or duration were observed (Table 2.1) (see Table 2.4 for full statistical analysis).

Non-pathologically aged mice display reduced arousal during the active phase.

Assessment of qEEG (quantitative electroencephalography) spectral changes during each sleep state (wake, NREM sleep and REM sleep) was performed to understand the deficits observed with non-pathological aging. During the active and inactive phases, a shift to lower powers was observed during wake (active: age, p=0.0006; frequency and age x frequency, p<0.0001; inactive: age, p=0.1383; frequency and age x frequency, p<0.0001) (Figure 2.3A and E). During the active phase, a consistent reduction in gamma power, a correlate of arousal was also observed (age, p<0.0001; time and age x time interaction, both p=0.0070) (Figure 2.3C); however, no change was seen during the inactive phase (age, p=0.4365; time and time x age interaction, both p=0.7044) (Figure 2.3H). During NREM sleep in the active phase, there was an increase in gamma power (age, p=0.1320; frequency and age x frequency, p<0.0001) (Figure

2.3B), with increased delta power (SWA) during NREM sleep in aged mice at the start of the active phase (age, p=0.5943; time and age x time, p<0.0001) (Figure 2.3D). During the inactive phase, there were no changes during NREM sleep (age, p=0.2606; frequency and age x frequency, p<0.0001) (Figure 2.3D) with no changes in delta power (SWA) during NREM sleep (age, p=0.09673; frequency and age x frequency, p<0.0001) (Figure 2.3I). During REM sleep, changes were observed in gamma power (age, p=0.7072; frequency and age x frequency,

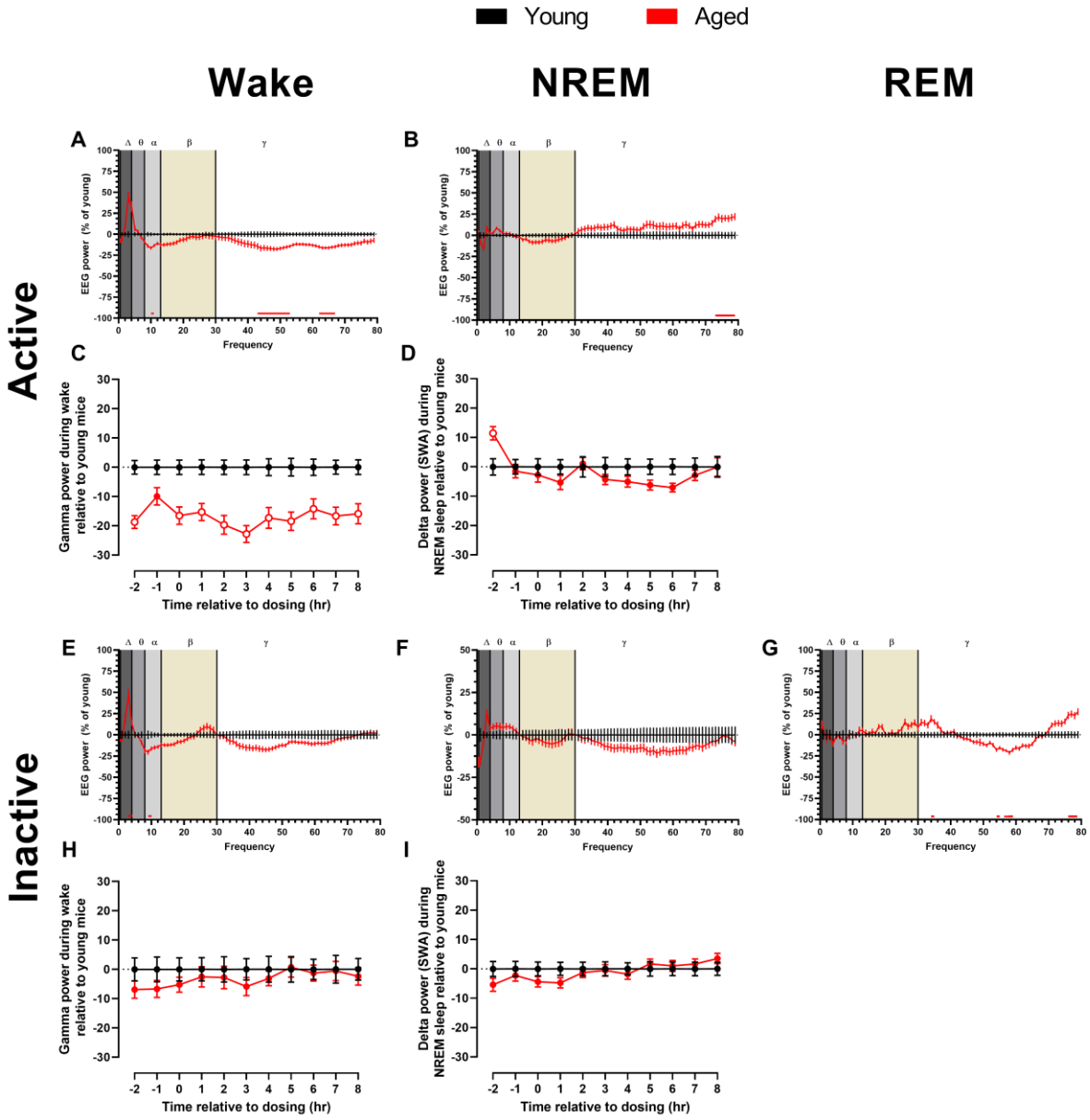

Figure 2.3. Non-pathologically aged mice displayed reduced arousal in the active phase compared to young mice. Shown is the relative spectral power in non-pathologically aged (19-20-month) mice normalized to young (3-4-month) mice from 0.5-80Hz during wake (A, E), NREM sleep (B, F), and REM sleep (G) during ZT 1-2 (drug baseline) in the active phase (A, B) and the inactive phase (E-G). Also shown are relative gamma power during wake normalized to young mice in 1-hour bins (C, H) and relative delta power (SWA activity, 0.5-4Hz) during NREM sleep normalized to young mice in 1-hour bins (D, I) in young and non-pathologically aged mice across the active phase (C, D) and the inactive phase (H, I). During the active phase, non-pathologically aged mice displayed a main effect of age and an age x frequency interaction on relative spectral power during wake with a reduction in relative power in alpha and gamma frequencies (A). During NREM sleep, non-pathologically aged mice displayed an age x frequency interaction with an increase in relative gamma power (B). Non-pathologically aged mice showed an age-related reduction in gamma power across the active phase (C) and no change in SWA during NREM sleep (D). In the inactive phase, non-pathologically aged mice displayed an age x frequency interaction with increased relative delta and reduced relative alpha power during wake (E); while during NREM sleep, no age-related changes were observed (F). During REM sleep, an age x frequency interaction was observed and non-pathologically aged mice displayed increased relative power at 34 and 76-79Hz and reduced relative power at 54 and 56-59Hz (G). During wake, no change in gamma power was seen (H), and no change in SWA during NREM sleep was observed (I). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A, B, E-G) and means ± S.E.M. in 1-hour bins (C, D, H, I), n=27-28/group. Solid bars at the bottom of the graph indicate $p<0.05$ compared to young (A, B, E, G). Open circles indicate $p<0.05$ (C, D) (all RM 2-way ANOVA matching by time followed by Sidak's test). See table 2.4 for full statistical analysis.

$p<0.0001$) (Figure 2.3G).

Xanomeline promoted wake and reversed wake fragmentation in non-pathologically aged mice in the active phase.

To examine the effects of the direct-acting M_1/M_4-preferring orthosteric mAChR agonist xanomeline on sleep/wake architecture in young and non-pathologically aged mice, we dosed mice with vehicle or xanomeline 2-3 hours into the active phase. Young mice displayed increased wake at ZT 18 following administration with the 10 mg/kg dose of xanomeline, while the 30 mg/kg dose of xanomeline increased wake at ZT 14 and 0 with a rebound decreased wake at ZT 20 (dose, time, and dose x time interaction, all $p<0.0001$) (Figure 2.4A). Both the 3 and 10 mg/kg doses of xanomeline produced increased total wake from ZT 12-24 and 30 mg/kg produced increased total wake from ZT 0-12 (dose and time, both $p<0.0001$; dose x time interaction, $p=0.0002$) in the young mice (Figure 2.4D). Due to the increased wake, subsequent decreased NREM sleep was seen at ZT 18 with the 10 mg/kg dose of xanomeline and at ZT 14 and 0 with the 30 mg/kg dose of xanomeline in the young mice. A rebound increased NREM sleep was observed at ZT 20 with the 30 mg/kg dose of xanomeline (dose, time, and dose x time interaction, all $p<0.0001$) (Figure 2.4B). There was decreased total NREM sleep at all doses of xanomeline

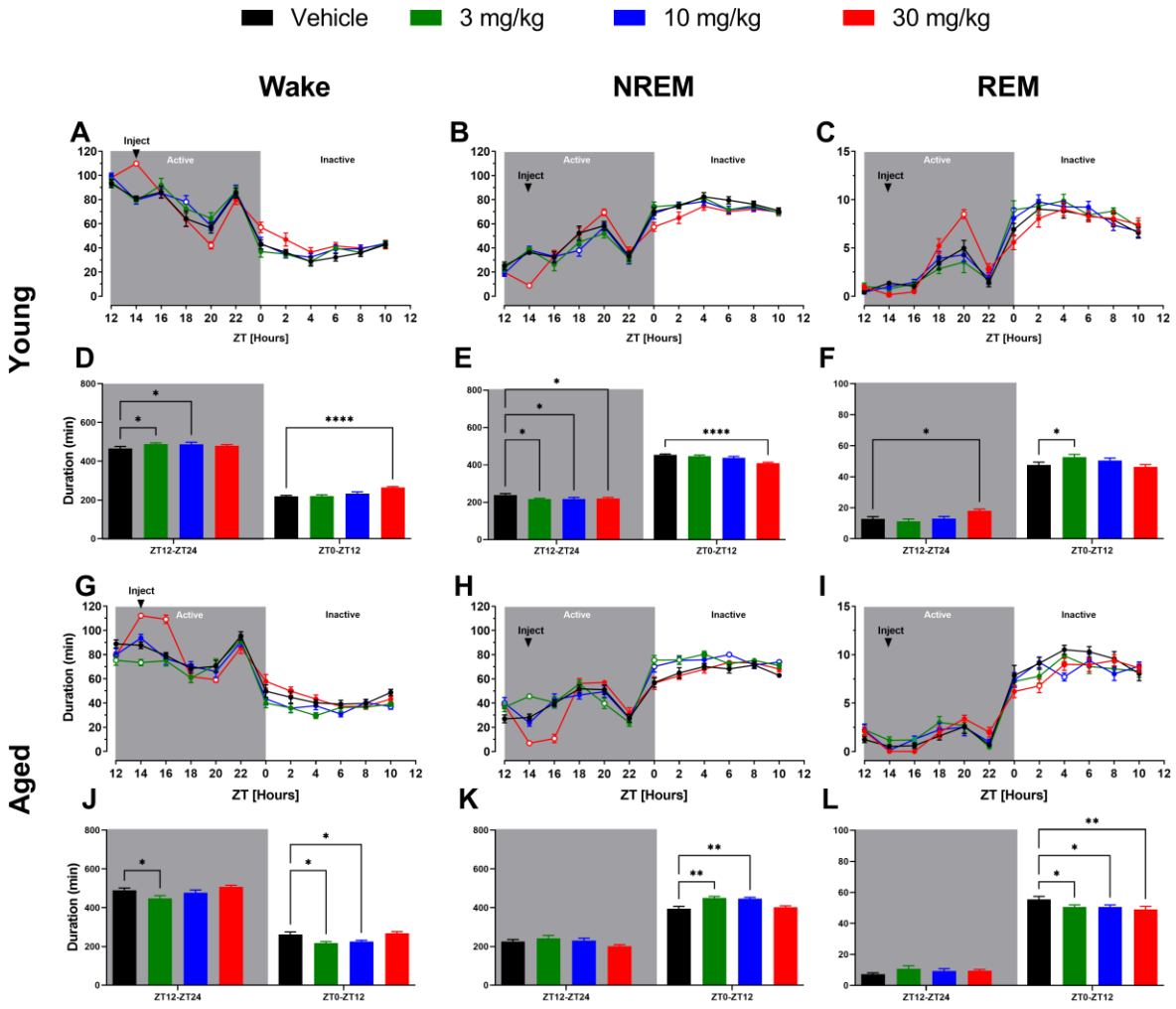

Figure 2.4. Xanomeline displayed wake promotion in the active phase in young and non-pathologically aged mice. Shown is the duration of time spent in wake (A, D, G, J), NREM sleep (B, E, H, K) and REM sleep (C, F, I, L) in young (A-F) and non-pathologically aged (G-L) mice following xanomeline administration 2 hours into the active phase (see arrowhead). In young mice, 30 mg/kg xanomeline produced an initial increase in wake, followed by a rebound decrease in wake; while 10 mg/kg xanomeline produced increased wake (A). Increased total wake over the 12 hours of the active phase was observed at 3 and 10 mg/kg, while 30 mg/kg producing increased total wake in the subsequent inactive phase (D). 30 mg/kg xanomeline produced decreased NREM sleep following dosing with a rebound increased NREM sleep, and 10 mg/kg produced decreased NREM sleep (B), 3-30 mg/kg produced reduced NREM sleep during the active phase with the effects at 30 mg/kg extending into the subsequent inactive phase (E). 30 mg/kg xanomeline increased REM sleep following dosing (C), with an overall increase in REM sleep observed during the active phase following dosing with 30 mg/kg xanomeline, while 3 mg/kg xanomeline produced increased REM during the subsequent inactive phase (F). In non-pathologically aged mice 30 mg/kg xanomeline produced increased wake and 3 mg/kg produced reduced wake following dosing (G), 3 mg/kg produced reduced total wake in the active and subsequent inactive phases and 10 mg/kg produced decreased wake in the inactive phase (J). 30 mg/kg reduced NREM sleep following dosing, and 3 mg/kg increased NREM sleep following dosing, with 3 and 10 mg/kg producing increased NREM sleep across the inactive phase (H). This resulted in an increased total NREM sleep at 3 and 10 mg/kg in the inactive phase following active phase dosing (K). Xanomeline had no effect on REM sleep immediately following active phase dosing, but all doses reduced REM sleep in the inactive phase (I and L). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA matching by both factors followed by Dunnett's test), or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ compared to vehicle (RM 1-way ANOVA followed by Dunnett's test), n=14/group; See table 2.4 for full statistical analysis.

from ZT 12-24 and decreased total NREM sleep at the 30 mg/kg dose of xanomeline from ZT 0-12 (dose and time, both p<0.0001; dose x time interaction, p=0.0012) in the young mice (Figure

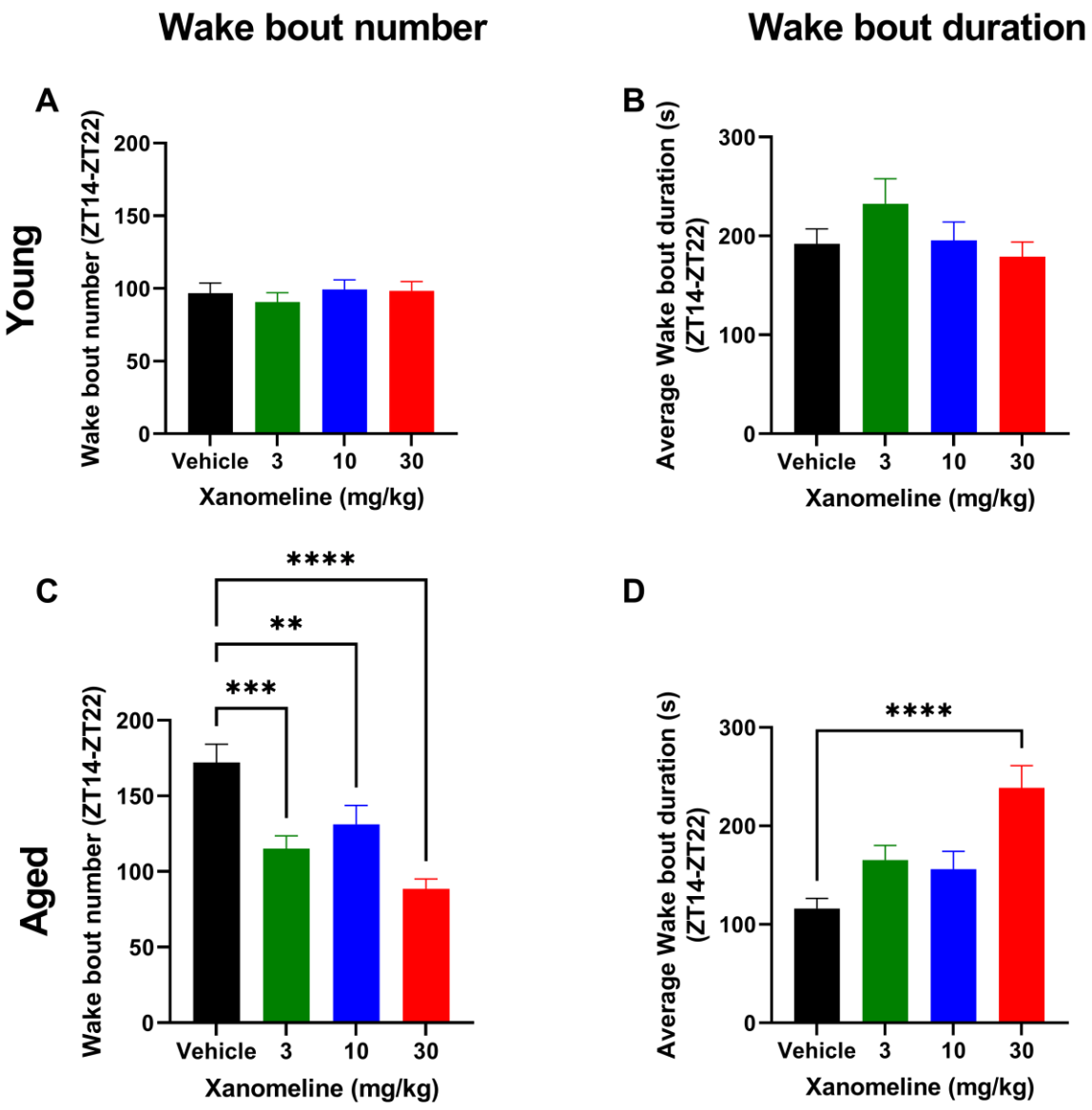

Figure 2.5. Xanomeline reduced wake bout number and increased wake bout duration during the active phase in non-pathologically aged mice. Shown is the average wake bout number (A, C) and the average wake bout duration (B, D) in young (A, B) and non-pathologically aged (C, D) mice during the 8-hours following dosing in the active phase. Xanomeline has no effect on wake bout number (A) or duration (B) when dosed in the active phase in young mice. In non-pathologically aged mice xanomeline dose dependently reduced wake bout number (C) and increased wake bout duration (D). Data are expressed as overall means ± S.E.M., n=14/group. ** indicates p<0.01, *** p<0.001 and **** p<0.0001 compared to vehicle (RM 1-way ANOVA followed by Dunnett's test). See table 2.4 for full statistical analysis

2.4E). The 3 mg/kg dose of xanomeline increased REM sleep at ZT 0 and the 30 mg/kg dose of xanomeline increased REM sleep at ZT 20 (dose, p=0.1296; time and dose x time interaction, both p<0.0001) in the young mice (Figure 2.4C). Increased total REM sleep was observed from ZT 12-24 following dosing with the 30 mg/kg dose of xanomeline and from ZT 0-12 at the 3 mg/kg dose of xanomeline (dose, p=0.1296; time and dose x time interaction, both p<0.0001) in the young mice (Figure 2.4F).

In non-pathologically aged mice, the 3 mg/kg dose of xanomeline decreased wake at ZT 14, with decreased wake also observed prior to dosing at ZT 12; while the 10 mg/kg dose of xanomeline decreased wake at ZT 10. In non-pathologically aged mice, the 30 mg/kg dose of xanomeline produced a more extended increase in wake at ZT 14 and 16 than observed in young mice. In addition, rebound decreased wake was observed at ZT 20 when xanomeline was dosed at 30 mg/kg (dose, time, and dose x time interaction, all p<0.0001) in the non-pathologically aged mice (Figure 2.4G). Total wake from ZT12-24 was reduced following administration of a 3 mg/kg dose of xanomeline, and total wake from ZT 0-12 was reduced following dosing with 3 and 10 mg/kg doses of xanomeline (dose and time, both p<0.0001; dose x time interaction, p=0.6602) in the non-pathologically aged mice (Figure 2.4J). The 3 mg/kg dose of xanomeline produced increased NREM sleep at ZT 14, 0 and 2 following dosing, with decreased NREM sleep at ZT 20. Xanomeline dosed at 10 mg/kg increased NREM sleep at ZT 0, 2, 6 and 10, with increased NREM also observed prior to dosing at ZT 12 in the non-pathologically aged mice. The 30 mg/kg dose of xanomeline produced decreased NREM sleep following dosing at ZT 14 and 16 (dose, time, and dose x time interaction, all p<0.0001) (Figure 2.4H). The 3 and 10 mg/kg doses of xanomeline increased total NREM sleep from ZT 0-12 (dose and time, both p<0.0001; dose x time interaction, p=0.1754) in the non-pathologically aged mice (Figure 2.4K). The dose of 10 mg/kg of xanomeline reduced REM sleep at ZT 4, while 30 mg/kg dose of xanomeline reduced REM sleep at ZT 2 (dose, p=0.3523; time, p<0.0001 and dose x time interaction, p=0.0035) (Figure 2.4I). All doses of xanomeline (3, 10 and 30 mg/kg) reduced total REM sleep from ZT 0-12 (dose,

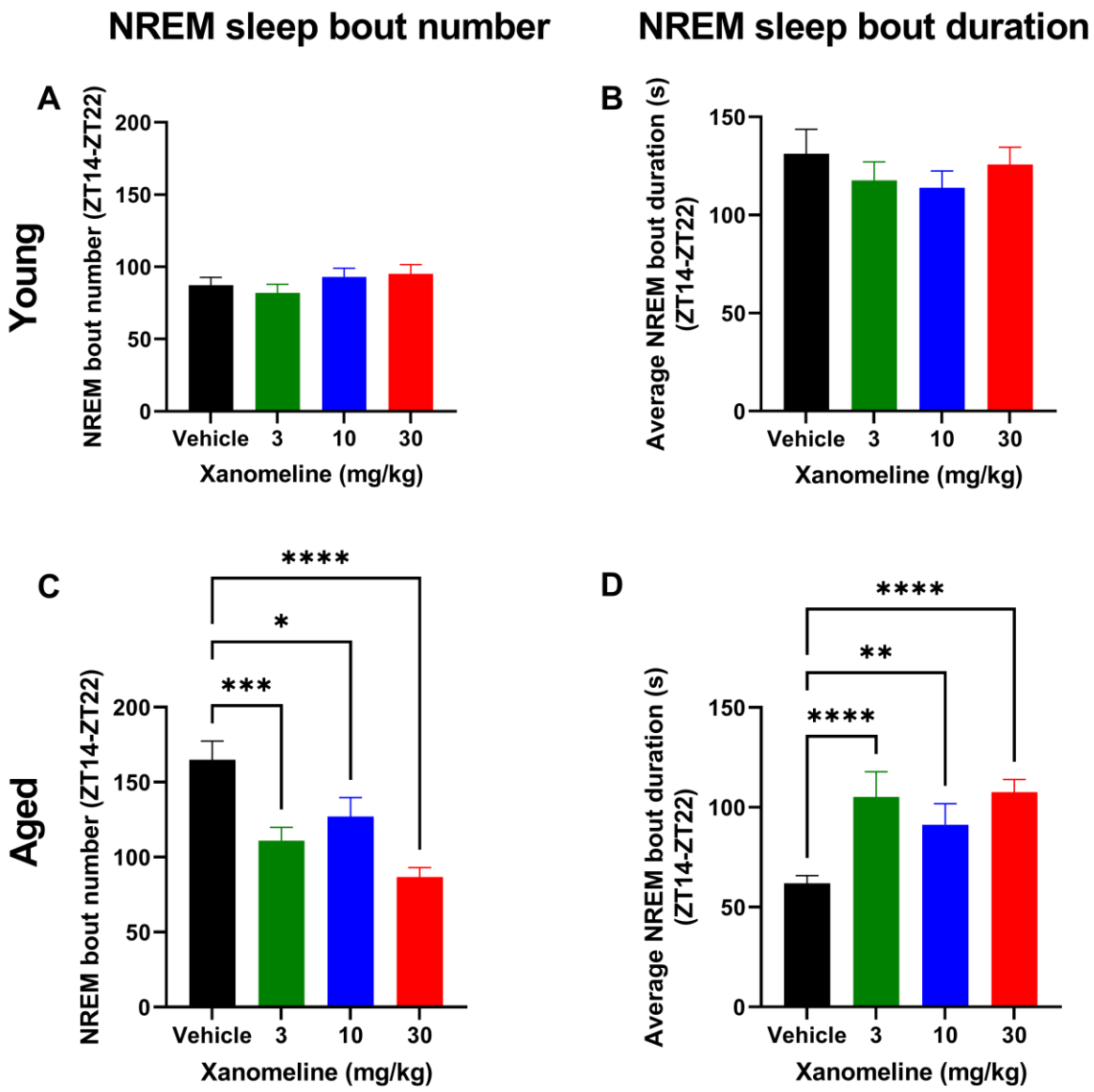

Figure 2.6. Xanomeline reduced NREM bout number and increased NREM bout duration in the active phase in non-pathologically aged mice. Shown is the average NREM sleep bout number (A, C) and the average NREM sleep bout duration (B, D) in young (A, B) and non-pathologically aged (C, D) mice for 8 hours following dosing in the active phase. Xanomeline had no effect on NREM sleep bout number or duration when dosed in the active phase in young mice (A and B). In non-pathologically aged mice xanomeline dose dependently reduced NREM sleep bout number (C) and increased NREM sleep bout duration (D). Data are expressed as overall means ± S.E.M., n=14/group. ** indicates p<0.01, *** p<0.001 and **** p<0.0001 compared to vehicle (RM 1-way ANOVA followed by Dunnett's test). See table 2.4 for full statistical analysis.

p=0.3523; time, p<0.0001; dose x time interaction, p=0.0033) in the non-pathologically aged mice (Figure 2.4L).

Given the wake-promoting effects of xanomeline and age-related increase in wake fragmentation, we investigated the effects of xanomeline on wake fragmentation during the active phase in the young and non-pathologically aged mice. Xanomeline had no effect on wake bout number (no main effect of dose, p=0.6576) or wake bout duration (no main effect of dose, p=0.1084) from ZT 14-22 in young mice (Figure 2.5A and B). However, in non-pathologically aged mice, all doses of xanomeline reduced wake bout number (main effect of dose, p<0.0001), and the 30 mg/kg dose of xanomeline increased wake bout duration (main effect of dose, p<0.0001) from ZT 14-22 (Figure 2.5C and D). Similar effects were observed on NREM sleep bout duration and number: xanomeline had no effect in young mice (NREM sleep bout duration, p=0.3031; NREM sleep bout number, p=0.3956) (Figure 2.6A and B); while in non-pathologically aged mice all doses of xanomeline produced decreased NREM bout number (main effect of dose, p<0.0001) and increased NREM bout duration (main effect of dose, p<0.0001) (Figure 2.6C and D).

Donepezil had no effect on wake in non-pathologically aged mice during the active phase.

Next, we assessed the effects of donepezil, an AChEI approved for the treatment of cognitive impairments in AD, on sleep/wake architecture. In young mice, the 0.1 mg/kg dose of donepezil increased wake at ZT 18 and the 3 mg/kg dose of donepezil increased wake at ZT 14 with a reduction in wake at ZT 16 (dose, p=0.3154; time, p<0.0001; dose x time interaction, p=0.0003) (Figure 2.7A), and no effect on overall wake from ZT 12-24 or ZT 0-12 (dose, p=0.3154; time, p<0.0001; dose x time interaction, p=0.2740) (Figure 2.7D). In young mice, the 0.1 mg/kg dose of donepezil decreased NREM sleep at ZT 18, and the 3 mg/kg dose of donepezil decreased NREM sleep at ZT 14 with an increase in NREM sleep at ZT 16. Additionally, increased NREM sleep was seen at the 1 mg/kg dose of donepezil prior to dosing (dose, p=0.4587; time and dose x time interaction, both p=0.0001) (Figure 2.7B), with no effect on total NREM sleep observed at any dose from ZT 12-24 or 0-12 (dose, p=0.4587; time, p<0.0001; dose x time interaction, p=0.1824) (Figure 2.7E). In young mice, the 0.1 and 0.3 mg/kg doses of donepezil decreased REM sleep at ZT 18 (dose, p=0.2762; time, p<0.0001; dose x time interaction, p=0.0216) (Figure

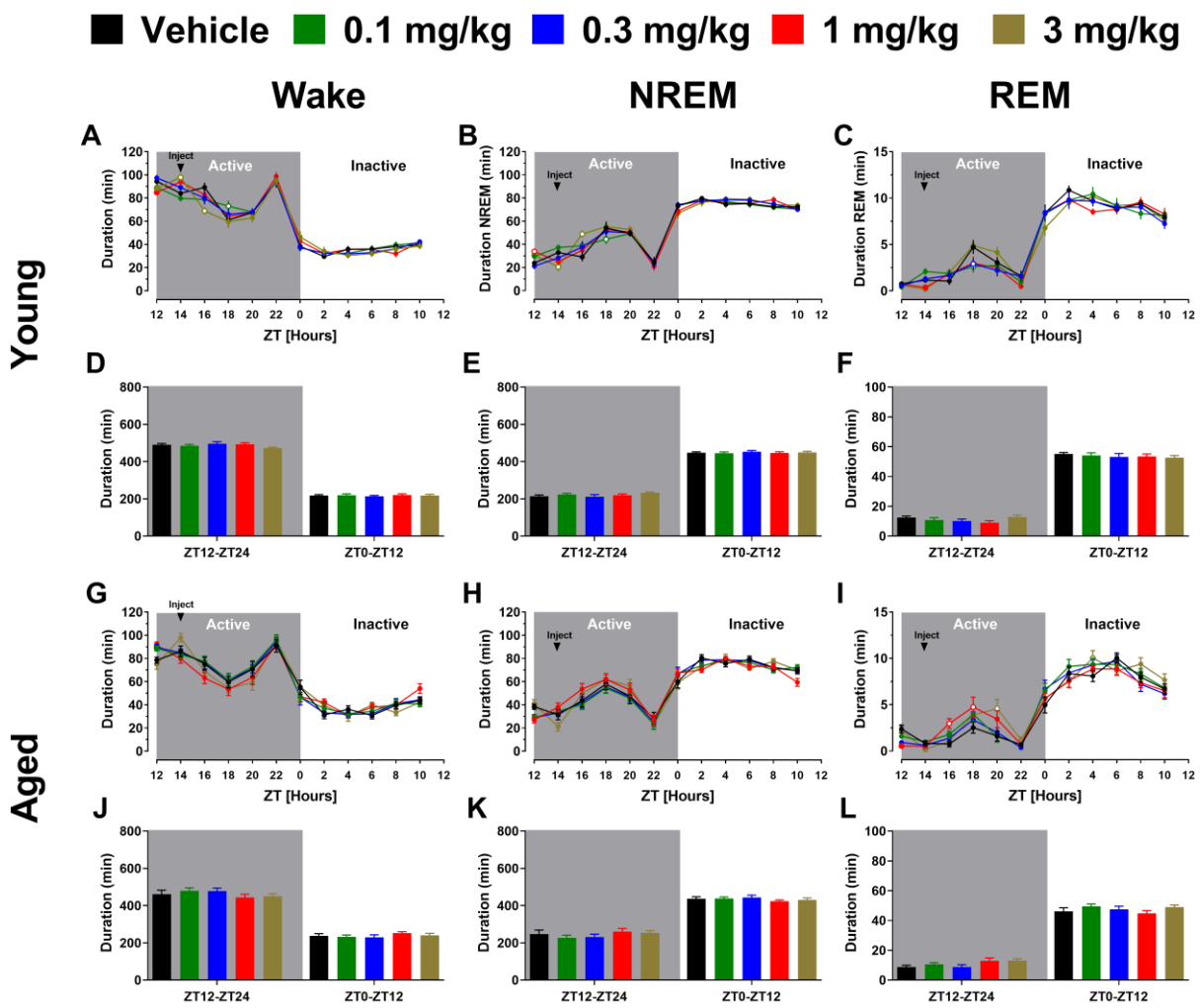

Figure 2.7. Donepezil had no effect on wake in the active phase in non-pathologically aged mice. Shown is the duration of time spent in wake (A, D, G, J), NREM sleep (B, E, H, K) and REM sleep (C, F, I, L) in young (A-F) and non-pathologically aged (G-L) mice following donepezil administration 2 hours into the active phase (see arrowhead). In young mice, 3 mg/kg of donepezil produced increased wake followed by a reduction in wake (A), with no effect on overall wake in the active phase (D). NREM sleep decreased following dosing with 3 mg/kg of donepezil before increasing (B), and no effect on overall NREM sleep during the active phase (E). 0.1 and 0.3 mg/kg donepezil reduced REM sleep following dosing (C), with no effect on overall REM sleep (F). In non-pathologically aged mice there was no dose related effect observed on wake (G, J) or NREM sleep (H, K). A dose-related increase in REM sleep was observed with 1 and 3 mg/kg donepezil (I), although no effect on total REM sleep was seen (L). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA matching by both factors followed by Dunnett's test), or 12-hour bins (D-F, J-L), n=14/group; See table 2.4 for full statistical analysis.

2.7C), with no effect observed on overall REM sleep from ZT 12-24 and ZT 0-12 (dose, $p=0.2762$; time, $p<0.0001$; dose x time interaction, $p=0.4230$) (Figure 2.7F).

In non-pathologically aged mice, donepezil had no effect on wake or NREM sleep when assessed as 2-hour epochs (wake: dose, $p=0.5692$; time, $p<0.0001$; dose x time interaction,

Table 2.2. Donepezil increased NREM bout number in young mice in the active phase.

		Young				Aged			
		Wake		NREM		Wake		NREM	
		Bout number (SEM), ZT12-14	Average bout duration, ZT12-14 (s) (SEM)	Bout number (SEM), ZT12-14	Average bout duration, ZT12-14 (s) (SEM)	Bout number (SEM), ZT12-14	Average bout duration, ZT12-14 (s) (SEM)	Bout number (SEM), ZT12-14	Average bout duration, ZT12-14 (s) (SEM)
Donepezil (mg/kg)	Vehicle	97.86 (5.384)	194.0 (12.34)	87.93 (4.686)	117.0 (7.916)	124.6 (12.32)	166.1 (21.20)	118.2 (12.47)	100.4 (11.81)
	0.1	106.9 (3.741)	172.1 (9.948)	100.6 (4.046)	102.7 (3.714)	119.4 (13.12)	203.1 (48.01)	112.4 (12.56)	105.5 (11.06)
	0.3	97.93 (3.679)	190.0 (8.692)	89.14 (3.430)	113.8 (6.711)	136.5 (13.07)	149.7 (17.10)	130.3 (12.97)	87.93 (9.329)
	1	104.2 (5.233)	183.8 (13.85)	100.6 (5.366)	101.1 (4.077)	108.3 (11.22)	174.1 (21.63)	104.3 (10.63)	123.0 (9.635)
	3	107.6 (4.579)	160.2 (8.768)	105.1 (4.371)*	109.3 (4.910)	122.0 (8.971)	147.1 (15.45)	120.1 (9.290)	101.2 (6.153)
1-way ANOVA		$F_{4,52} = 1.263$	$F_{4,52} = 2.244$	$F_{4,52} = 3.765$	$F_{4,52} = 2.241$	$F_{4,52} = 9.779$	$F_{4,52} = 0.8950$	$F_{4,52} = 0.9223$	$F_{4,52} = 2.805$
P value		0.2963	0.0768	0.0092	0.0772	0.4277	0.4737	0.4581	0.0349

*p<0.05, Dunnetts multiple comparisons compared to vehicle condition

p=0.0002; NREM: dose, p=0.6304; time, p<0.0001; dose x time interaction, p=0.0002) (Figure 2.7G and H) or as total amount of wake or NREM sleep respectively from ZT 12-24 or ZT 0-12 (wake: dose, p=0.5692; time, p<0.0001; dose x time interaction, p=0.1623; NREM: dose, p=0.6304; time, p<0.0001; dose x time interaction, p=0.2095) (Figure 2.7J and K). In non-pathologically aged mice, the 1 mg/kg dose of donepezil increased REM sleep at ZT 16 and 18, and the 3 mg/kg dose of donepezil increased REM sleep at ZT 20 (dose, p=0.0033; time, p<0.0001; dose x time interaction p=0.0008) (Figure 2.7I), with a main effect of donepezil on total REM sleep between ZT 12-24 and ZT 0-12, but no effect at any specific dose on post hoc analysis (dose, p=0.0033; time, p<0.0001; dose x time interaction, p=0.1763) (Figure 2.7L).

When assessing wake bout fragmentation following dosing in the active phase in young and non-pathologically aged mice, donepezil produced no effect on wake bout number or average wake bout duration. However, dosing donepezil at 3 mg/kg in young mice during the active phase increased NREM sleep bout number with no effect on NREM sleep bout duration. In non-pathologically aged mice, donepezil had no effect on NREM sleep bout number, with a significant overall effect of dose on NREM sleep bout duration, but no significant effect at any dose following *post hoc* analysis (Table 2.2).

Xanomeline and donepezil promoted wake when dosed in the inactive phase.

Given the wake-promoting effects of xanomeline, we next assessed whether xanomeline and donepezil would be disruptive to sleep when dosed in the inactive phase. Xanomeline dose-dependently increased wake in young and non-pathologically aged mice when dosed in the inactive phase. In young mice, xanomeline produce the following dose-related changes in wake: 3 mg/kg dose of xanomeline increased wake at ZT 4, 10 mg/kg dose of xanomeline increased wake at ZT 2 and 4, with rebound reductions observed at ZT 18, and the 30 mg/kg dose of xanomeline increased wake at ZT 2 and 4 with rebound decreased wake seen at ZT 14, 16 and 18 (dose, $p=0.0006$; time and dose x time interaction, both $p<0.0001$) (Figure 2.8A). There was also an increase in total wake observed over 12 hours from ZT 0-12 after the 10 and 30 mg/kg

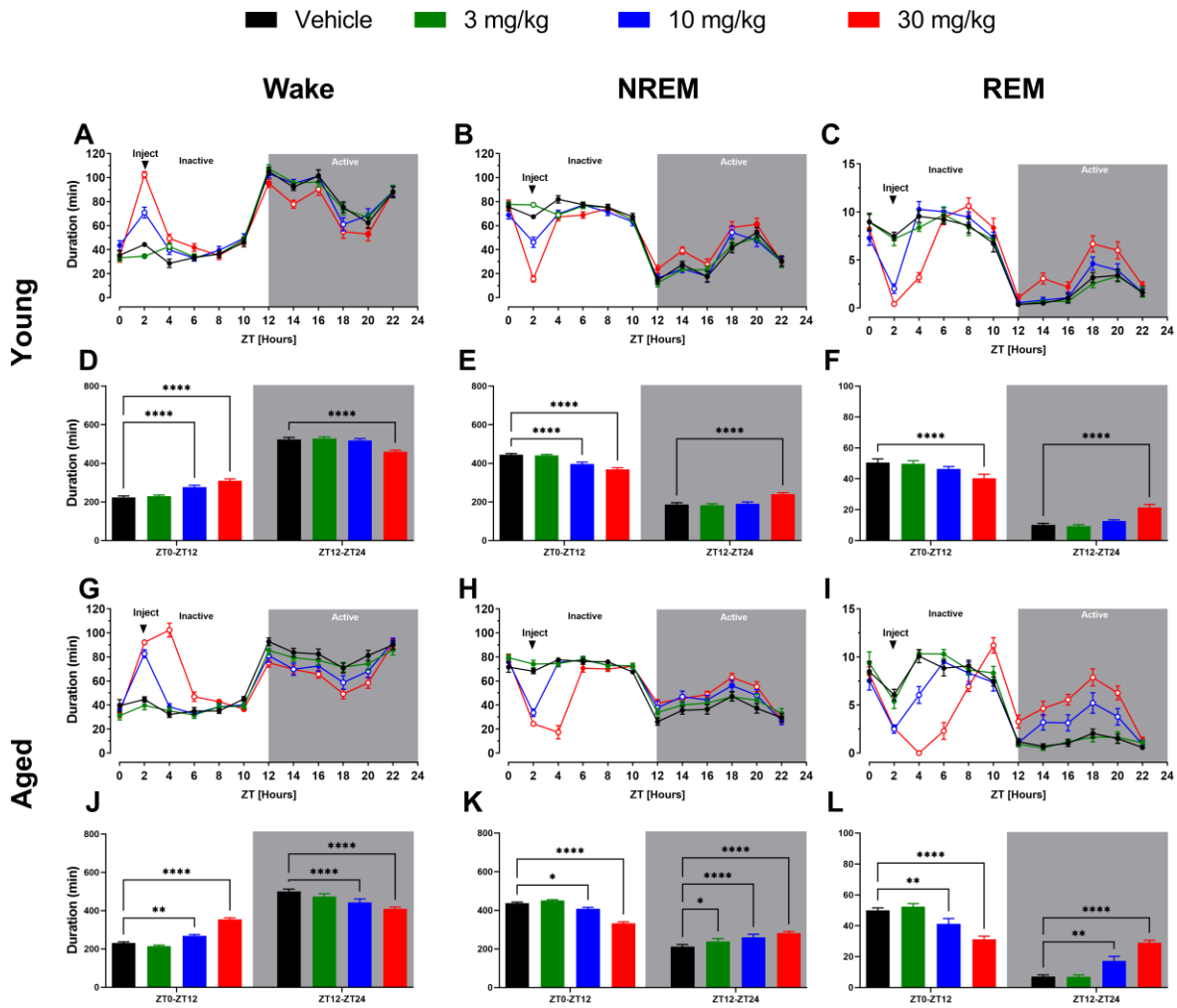

Figure 2.8. Xanomeline increased wakefulness in the inactive phase in young and non-pathologically aged mice. Shown is the duration of time spent in wake (A, D, G, J), NREM sleep (B, E, H, K) and REM sleep (C, F, I, L) in young (A-F) and non-pathologically aged (G-L) mice following xanomeline administration 2 hours into the inactive phase (see arrowhead). In young mice, 10 and 30 mg/kg xanomeline produced increased wake with a subsequent rebound decreased wake in the active phase, and 3mg/kg produced a transient wake increase (A), with increased total wake over the 12 hours of the inactive phase observed at 10 and 30 mg/kg, and 30 mg/kg also producing decreased wake in the subsequent active phase (D). 10 and 30 mg/kg xanomeline produced decreased NREM sleep following dosing, with a rebound increase observed in the subsequent active phase, and 3mg/kg produced increased NREM sleep at ZT 2 and decreased NREM sleep at ZT 4 (B), 10 and 30 mg/kg produced reduced NREM sleep during the inactive phase with increased NREM sleep observed at 30 mg/kg in the subsequent active phase (E). 10 and 30 mg/kg xanomeline decreased REM sleep following dosing, with rebound increased REM sleep seen in the 30 mg/kg group (C). An overall decrease in total REM sleep was seen during the inactive phase following dosing with 30 mg/kg xanomeline and increased total REM sleep was observed in the subsequent active phase (F). In non-pathologically aged mice 10 and 30 mg/kg xanomeline produced increased wake following dosing with a rebound decreased wake in the subsequent active phase (G), 10 and 30 mg/kg xanomeline increased total wake in the inactive phase and reduced total wake in the subsequent active phase (J). 10 and 30 mg/kg xanomeline reduced NREM sleep following dosing with a subsequent rebound increased NREM sleep in the active phase (H). This resulted in a decreased total NREM sleep at 10 and 30 mg/kg in the inactive phase and increased NREM sleep at all doses in the subsequent active phase (K). Xanomeline 10 and 30 mg/kg reduced REM sleep following inactive phase dosing with subsequent rebound increased REM sleep (I). Total REM sleep was decreased in the inactive phase at 10 and 30 mg/kg xanomeline and increased in the subsequent active phase following 10 and 30 mg/kg xanomeline (L). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA matching by both factors followed by Dunnett's test), or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ compared to vehicle (RM 1-way ANOVA followed by Dunnett's test), n=13-14/group; See table 2.4 for full statistical analysis.

doses of xanomeline and reduced total wake observed from ZT 12-24 with the 30 mg/kg dose of xanomeline (dose, $p=0.0006$; time, $p<0.0001$ and dose x time interaction, both $p<0.0001$) (Figure 2.8D). Consistent with wake promotion, NREM and REM sleep were reduced following dosing in the young mice. Xanomeline produced the following dose-related changes in NREM: the dose of 3 mg/kg of xanomeline reduced NREM sleep at ZT 4, the 10 mg/kg dose of xanomeline reduced NREM sleep at ZT 2 and 4 with rebound increased NREM sleep at ZT 18, and the 30 mg/kg dose of xanomeline decreased NREM sleep at ZT 2 and 4 with rebound increased NREM sleep observed at ZT 14, 16 and 18. Additionally, increased NREM sleep was observed at ZT 2 with 3 mg/kg xanomeline (dose, $p=0.0006$; time and dose x time interaction, both $p<0.0001$) in the young mice (Figure 2.8B). Total NREM sleep was decreased between ZT 0-12 following dosing with 10 and 30 mg/kg doses of xanomeline and NREM sleep was increased between ZT 12-24 following dosing with the 30 mg/kg dose of xanomeline (dose, $p=0.0006$; time and dose x time interaction, both $p<0.0001$) (Figure 2.8E). Xanomeline produced the following dose-related changes in REM in young mice: the 10 mg/kg dose of xanomeline decreased REM sleep at ZT 2 and the 30 mg/kg

dose of xanomeline decreased REM sleep at ZT 2 and 4 with a rebound increased REM sleep seen at ZT 8, 14, 18 and 20 (dose, p=0.7367; time and dose x time interaction, both p<0.0001) (Figure 2.8C). Reduced total REM sleep was observed with the 30 mg/kg dose of xanomeline between ZT 0-12 with a rebound increased total REM sleep between ZT 12-24 (p=0.7367; time and dose x time interaction, both p<0.0001) in the young mice (Figure 2.8F).

In non-pathologically aged mice, the following pronounced wake-promoting effects of xanomeline were observed: the 10 mg/kg dose of xanomeline increased wake at ZT 2, with rebound decreased wake at ZT 12, 14, 18 and 20, while the dose of 30 mg/kg of xanomeline increased wake at ZT 2, 4 and 6 with rebound decreased wake seen at ZT 10, 12, 14, 16, 18 and 20 (dose, time, and dose x time interaction, all p<0.0001) (Figure 2.8G). Total wake following dosing between ZT 0-12 was also increased with the 10 and 30 mg/kg doses of xanomeline and a rebound decrease in total wake was observed between ZT 12-24 (dose and dose x time interaction, both p<0.0001; time, p=0.0024) in the non-pathologically aged mice (Figure 2.8J). With the observed increased wake in the non-pathologically aged mice following dosing with xanomeline, the following decreases in NREM sleep were seen: the 10 mg/kg dose of xanomeline reduced NREM sleep at ZT 2 with rebound increased NREM sleep seen at ZT 12,14 and 20; while the 30 mg/kg dose of xanomeline decreased NREM sleep at ZT 2 and 4 with rebound increased NREM sleep seen at ZT 12, 14, 16, 18 and 20 (dose, p=0.0011; time and dose x time interaction, both p<0.0001) (Figure 2.8H). Total NREM sleep from ZT 0-12 was reduced following dosing with the 10 and 30 mg/kg doses of xanomeline, while increased NREM sleep from ZT 12-24 was observed at the 3, 10 and 30 mg/kg doses of xanomeline (dose, p=0.0011; time and dose x time interaction, both p<0.0001) (Figure 2.8K) in the non-pathologically aged mice. The following dose-related reductions in REM sleep were also observed with xanomeline in the non-pathologically aged mice: the 10 mg/kg dose of xanomeline reduced REM sleep at ZT 2 and 4, with rebound increased REM sleep observed at ZT 14, 16, 18 and 20; while the 30 mg/kg dose of xanomeline reduced REM sleep at ZT 2, 4, 6 and 8 with rebound increased REM sleep

observed at ZT 10, 12, 14, 16, 18 and 20 (dose, p=0.5442; time and dose x time interaction, both p<0.0001) (Figure 2.8L). Total REM sleep from ZT 0-12 was reduced following dosing with the 10 and 30 mg/kg doses of xanomeline with rebound increased REM sleep seen at both doses from ZT 12-24 (dose, p=0.5442; time and dose x time interaction, both p<0.0001) (Figure 2.8L) in the non-pathologically aged mice.

Similar to xanomeline, donepezil increased wake following dosing in the inactive phase in young and non-pathologically aged mice. In the young mice, the 3 mg/kg dose of donepezil increased wake at ZT 2 with rebound decreases in wake observed at ZT 12, 14 and 20 (dose, p=0.1761; time and dose x time interaction, both p<0.0001) (Figure 2.9A). Total wake was

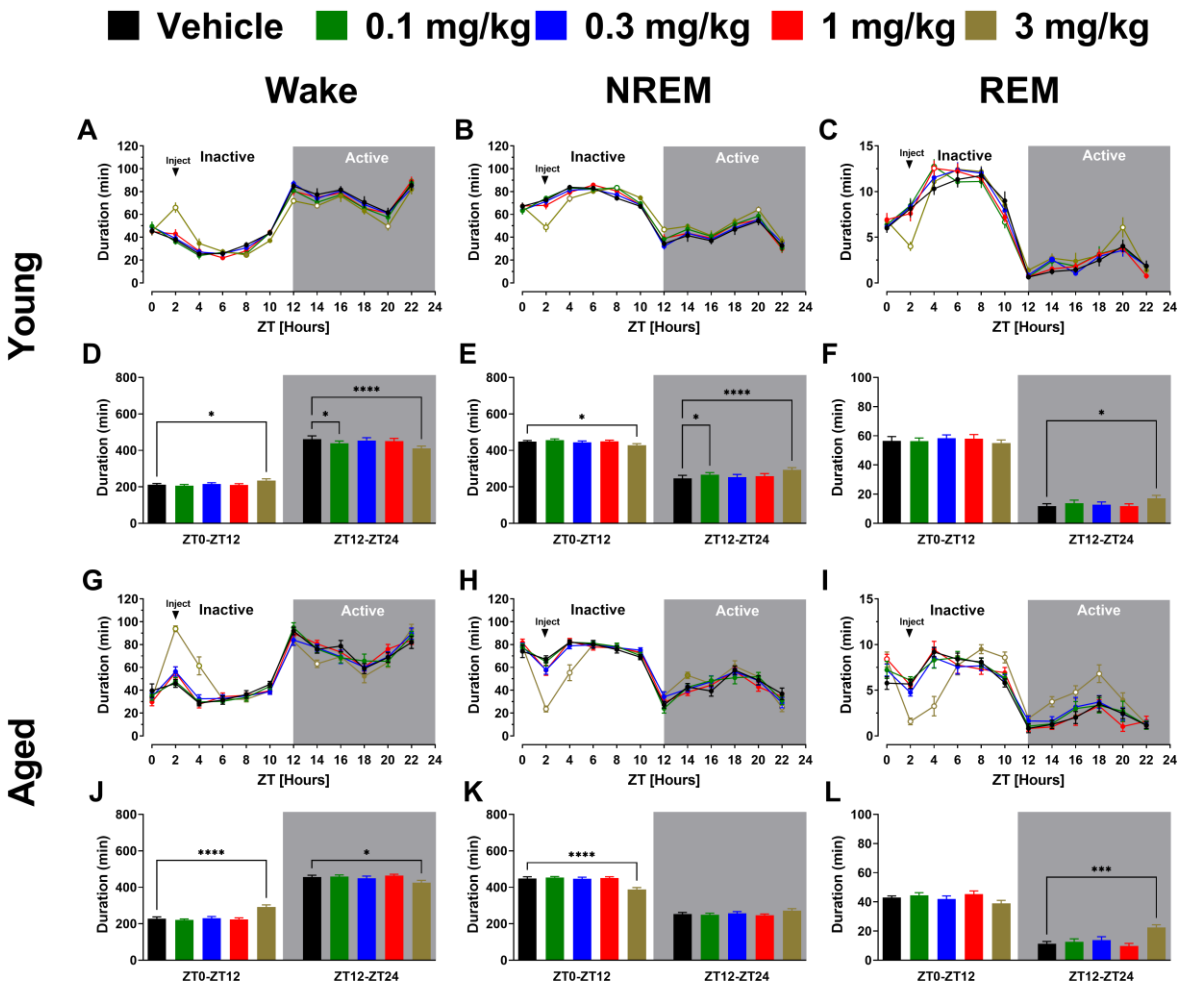

Figure 2.9. Donepezil increased wakefulness in the inactive phase in young and non-pathologically aged mice. Shown is the duration of time spent in wake (A, D, G, J), NREM sleep (B, E, H, K) and REM sleep (C, F, I, L) in young (A-F) and non-pathologically aged (G-L) mice following donepezil administration 2 hours into the inactive phase (see arrowhead). In young mice, 3 mg/kg donepezil increased wake following dosing, with decreased wake in the subsequent active phase (A). 3 mg/kg donepezil increased total wake in the inactive phase, and 0.1 and 3 mg/kg donepezil produced decreased total wake in the subsequent active phase (D). 3 mg/kg donepezil reduced NREM sleep following dosing, with subsequent rebound increased NREM sleep (B). 3 mg/kg donepezil produced decreased total NREM sleep in the inactive phase, 0.1 and 3 mg/kg increased NREM sleep in the subsequent active phase (E). 3 mg/kg donepezil reduced REM sleep following dosing, while 0.1 and 1 mg/kg increased REM sleep following dosing (C). There was no change in total REM sleep in the inactive phase following dosing, in the subsequent active phase increased REM sleep was observed following 3 mg/kg donepezil (F). In non-pathologically aged mice 3 mg/kg donepezil increased wake following dosing with rebound decreased wake in the subsequent active phase (G), this resulted in increased total wake during the inactive phase following 3 mg/kg donepezil dosing and reduced total wake in the subsequent active phase (J). 3 mg/kg donepezil produced reduced NREM sleep following dosing (H), with a reduction also seen in total NREM sleep during the inactive phase (K). 3 mg/kg donepezil produced an initial reduction in REM sleep, with subsequent rebound increased REM sleep (I), this resulted in an increased total REM sleep in the active phase following inactive phase dosing (L). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA matching by both factors followed by Dunnett's test), or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ compared to vehicle (RM 1-way ANOVA followed by Dunnett's test), n=13-14/group; See table 2.4 for full statistical analysis.

increased following dosing with donepezil at 3 mg/kg between ZT 0-12 and reduced wake was observed between ZT 12-24 following dosing with the 0.1 and 3 mg/kg doses of donepezil (dose, $p=0.1761$; time and dose x time interaction, both $p<0.0001$) (Figure 2.9D) in the young mice. The following dose-related changes in NREM sleep were also observed with donepezil in the young mice: the 0.1 mg/kg dose of donepezil increased NREM sleep at ZT 8, while the 3 mg/kg dose of donepezil decreased NREM sleep at ZT 2 and 4 with a rebound increased NREM sleep at ZT 12 and 20 (dose, $p=0.2016$; time and dose x time interaction, both $p<0.0001$) (Figure 2.9B). Total NREM sleep between ZT 0-12 was reduced by the 3 mg/kg dose of donepezil, and between ZT 12-24 total NREM sleep was increased following dosing with the 0.1 and 3 mg/kg doses of donepezil (dose, $p=0.1761$; time and dose x time interaction, both $p<0.0001$) (Figure 2.9E). In the young mice, the following dose-related changes in REM sleep were also observed with donepezil: REM sleep was increased at ZT 4 following dosing with the 0.1 and 3 mg/kg doses of donepezil and reduced following the 3 mg/kg dose of donepezil at ZT 2 with a rebound increased REM sleep at ZT 20 (dose, $p=0.7558$; time and dose x time interaction, both $p<0.0001$) (Figure 2.9C). Total REM sleep was unchanged between ZT 0-12, and total REM sleep was modestly increased with

the 3 mg/kg dose of donepezil between ZT 12-24 (dose, p=0.1761; time, p<0.0001; dose x time interaction, p=0.0417) (Figure 2.9F).

In the non-pathologically aged mice, the 3 mg/kg dose of donepezil increased wake at ZT 2 and 4 with reduced wake at ZT 14 (dose, p=0.0433; time and dose x time interaction, both p<0.0001) (Figure 2.9G). As shown in Figure 2.9J, overall wake was increased between ZT 0-12 and reduced between ZT 12-24 following administration of the 3 mg/kg dose of donepezil (dose, p=0.0433; time and dose x time interaction, both p<0.0001). The 3 mg/kg dose of donepezil also reduced NREM sleep at ZT 2 and 4 (dose, p=0.0090; time and dose x time interaction, both p<0.0001) (Figure 2.9H), with overall NREM sleep reductions between ZT 0-12 (dose, p=0.0433; time and dose x time interaction, both p<0.0001) in the non-pathologically aged mice (Figure 2.9K). The 3 mg/kg dose of donepezil decreased REM sleep at ZT 2 and 4, with rebound increased REM observed at ZT 10, 14, 16 and 18 in the non-pathologically aged mice. Additionally, an increase in REM sleep was observed at the 1 mg/kg dose of donepezil prior to dosing at ZT 0 (dose, p=0.0214; time and dose x time interaction, both p<0.0001) (Figure 2.9I). As shown in Figure 2.9L, there was no change in total REM sleep from ZT 0-12 following dosing

Table 2.3. Effects of xanomeline and donepezil on wake and NREM fragmentation during the inactive phase in young and non-pathologically aged mice.

		Young				Aged			
		Wake		NREM		Wake		NREM	
		Bout number, ZT2-10 (SEM)	Average bout duration, ZT2-10 (s), (SEM)	Bout number, ZT2-10 (SEM)	Average bout duration, ZT2-10 (s) (SEM)	Bout number, ZT2-10 (SEM)	Average bout duration, ZT2-10 (s), (SEM)	Bout number, ZT2-10 (SEM)	Average bout duration, ZT2-10 (s) (SEM)
Xanomeline (mg/kg)	Vehicle	100.3 (3.552)	77.83 (3.771)	101.1 (3.478)	189.9 (7.645)	105.9 (3.996)	79.65 (3.350)	106.5 (3.517)	174.4 (7.528)
	3	101.4 (4.343)	90.89 (4.846)	100.7 (4.489)	179.9 (8.768)	95.69 (3.834)	87.63 (4.086)	98.31 (3.795)	189.2 (7.952)
	10	100.4 (6.360)	101.1 (7.655)*	97.86 (6.557)	180.7 (9.972)	119.9 (10.75)	88.35 (9.693)	120.8 (10.78)	156.2 (11.45)
	30	93.79 (5.345)	126.0 (8.73)****	91.07 (5.538)	178.7 (9.147)	96.77 (6.678)	161.2 (14.95)****	95.69 (6.711)	141.9 (8.663)**
1-way ANOVA		$F_{3,39}$ = 0.5026	$F_{3,39}$ = 12.42	$F_{3,39}$ = 0.8155	$F_{3,39}$ = 0.4077	$F_{3,36}$ = 3.425	$F_{3,36}$ = 21.27	$F_{3,36}$ = 3.492	$F_{3,36}$ = 8.330
P value		0.6827	<0.0001	0.4931	0.7484	0.0272	<0.0001	0.0253	0.0002
Donepezil (mg/kg)	Vehicle	107.7 (3.864)	65.90 (3.341)	106.5 (4.281)	183.3 (8.886)	108.7 (5.486)	73.65 (4.136)	108.8 (5.634)	181.2 (9.980)
	0.1	98.46 (5.297)	69.40 (3.512)	99.08 (5.317)	204.0 (11.36)	116.1 (8.822)	72.66 (4.509)	116.6 (8.723)	172.1 (10.98)
	0.3	106.4 (17.99)	67.25 (3.278)	106.5 (5.021)	184.0 (9.716)	119.9 (9.167)	71.87 (4.036)	121.5 (8.928)	164.5 (11.68)
	1	96.62 (5.424)	72.83 (3.602)	95.23 (5.021)	209.1 (12.43)	109.6 (8.478)	75.43 (4.153)	110.9 (8.528)	182.8 (12.57)
	3	86.23 (4.008)**	91.93 (5.831)****	83.46 (3.640)***	226.4 (11.75)**	123.2 (8.584)	90.99 (8.022)	122.9 (8.563)	140.7 (8.501)*
1-way ANOVA		$F_{4,48}$ = 4.998	$F_{4,48}$ = 7.566	$F_{4,48}$ = 6.272	$F_{4,48}$ = 4.481	$F_{4,52}$ = 0.6461	$F_{4,52}$ = 0.2230	$F_{4,52}$ = 0.6724	$F_{4,52}$ = 3.364
P value		0.0019	<0.0001	0.0004	0.0037	0.6322	0.0784	0.6141	0.0160

*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, Dunnetts multiple comparisons compared to vehicle condition

with donepezil, while the 3 mg/kg dose of donepezil produced an increase in total REM sleep from ZT 12-24 (dose, p=0.0214; time, p<0.0001; dose x time interaction, p=0.0004) in the non-

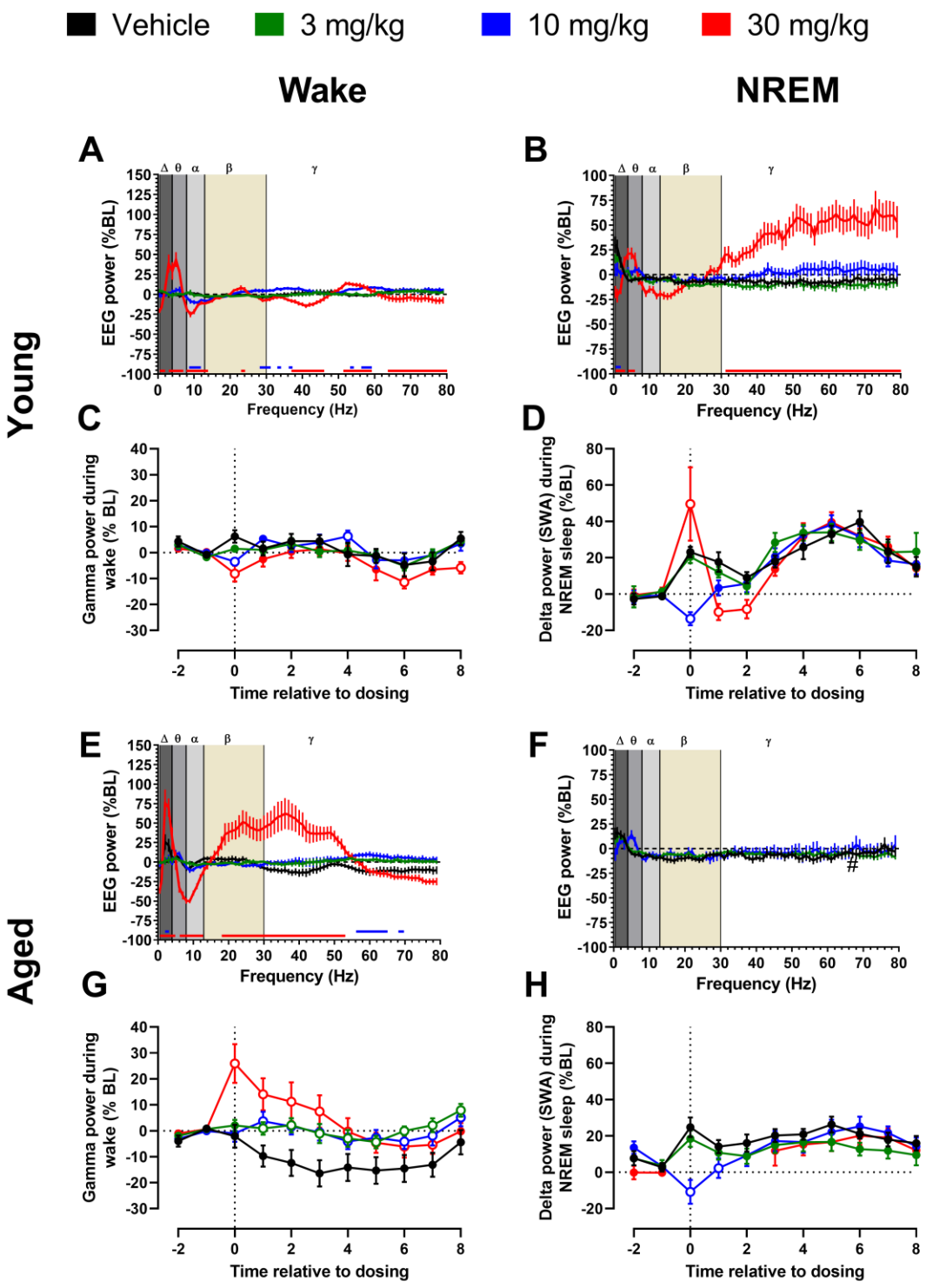

Figure 2.10. During the active phase, xanomeline produced dose dependent increases in arousal in non-pathologically aged mice and reduced delta power (SWA) during NREM sleep. Shown is the relative spectral power during wake (A, E) and NREM sleep (B, F) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline, gamma power during wake (C, G) and relative delta power (SWA) during NREM sleep (D, H), during the active phase in young (A-D) and non-pathologically aged (E-H) mice. In young animals during wake 1-2 hours post dose, 30 mg/kg xanomeline produced increased theta power and fluctuations across the gamma power range, both 10 and 30 mg/kg produced reduced alpha power (A). During NREM sleep epochs 1-2 hours post dose 30 mg/kg reduced delta power and increased theta and gamma power (B). 30 mg/kg xanomeline produced modest reductions in gamma power (C), and 10 and 30 mg/kg xanomeline reduced delta power (SWA) during NREM sleep following a transient increase at 30 mg/kg (D). In non-pathologically aged mice, 10 mg/kg xanomeline decreased delta power and increased gamma power during wake epochs, and 30 mg/kg xanomeline increased delta, beta and gamma power, and reduced theta and alpha power (E). Xanomeline had no effect on spectral power during NREM sleep, however there were fewer than 5 mice that exhibited NREM sleep during the analysis window at the 30 mg/kg dose. Xanomeline dose-dependently increased gamma power with increases seen at 3, 10 and 30 mg/kg during wake epochs (G) and produced reduced delta power (SWA) during NREM sleep at 10 mg/kg xanomeline, with no data available from 0-2 hours post dose in the 30 mg/kg group due to insufficient mice displaying NREM sleep (H). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A, B, E, F) and means ± S.E.M. in 1-hour bins. (C, D, G, H), n=12-14/group, all time points in time courses contain n=5-14 mice (C, D, G, H). Groups with fewer than 14 are due to not all mice displaying NREM sleep, # indicates fewer than 5 mice displayed NREM sleep in the 30 mg/kg dose group, so this was excluded. Solid bars indicate $p<0.05$ compared to vehicle (A, B, E, F), open symbols indicate $p<0.05$ compared to vehicle (C, D, G, H) (RM 2-way ANOVA matching by both factors followed by Dunnett's test for A, C, E, G and RM mixed effect model matching by both factors followed by Dunnett's test for B, D, F, H). See table 2.4 for full statistical analysis.

pathologically aged mice (Figure 2.9L).

As shown in Table 2.3, when assessing wake and NREM sleep fragmentation following dosing in the inactive phase, xanomeline produced dose-dependent increases in average wake bout duration in young animals and increased wake and decreased NREM sleep bout duration in non-pathologically aged mice. In comparison, donepezil at the 3 mg/kg dose reduced wake and NREM sleep bout number and increased average wake and NREM sleep bout duration in young mice; while in the non-pathologically aged mice, this dose of donepezil decreased average NREM sleep bout duration (Table 2.3).

Xanomeline increased arousal in non-pathologically aged mice during the active phase, while donepezil had no effect.

Next, we evaluated potential state-dependent changes in qEEG with xanomeline treatment in both young and non-pathologically aged mice. In the young mice, the 30 mg/kg dose of xanomeline produced increased theta and reduced alpha power (dose, $p=0.0725$; time and dose x time interaction, both $p<0.0001$) (Figure 2.10A). Modest reductions in gamma power

during wake following dosing at the 10 and 30 mg/kg doses of xanomeline were also observed in the young mice (dose, p=0.1824; time p<0.0001 and dose x time interaction p=0.0014) (Figure

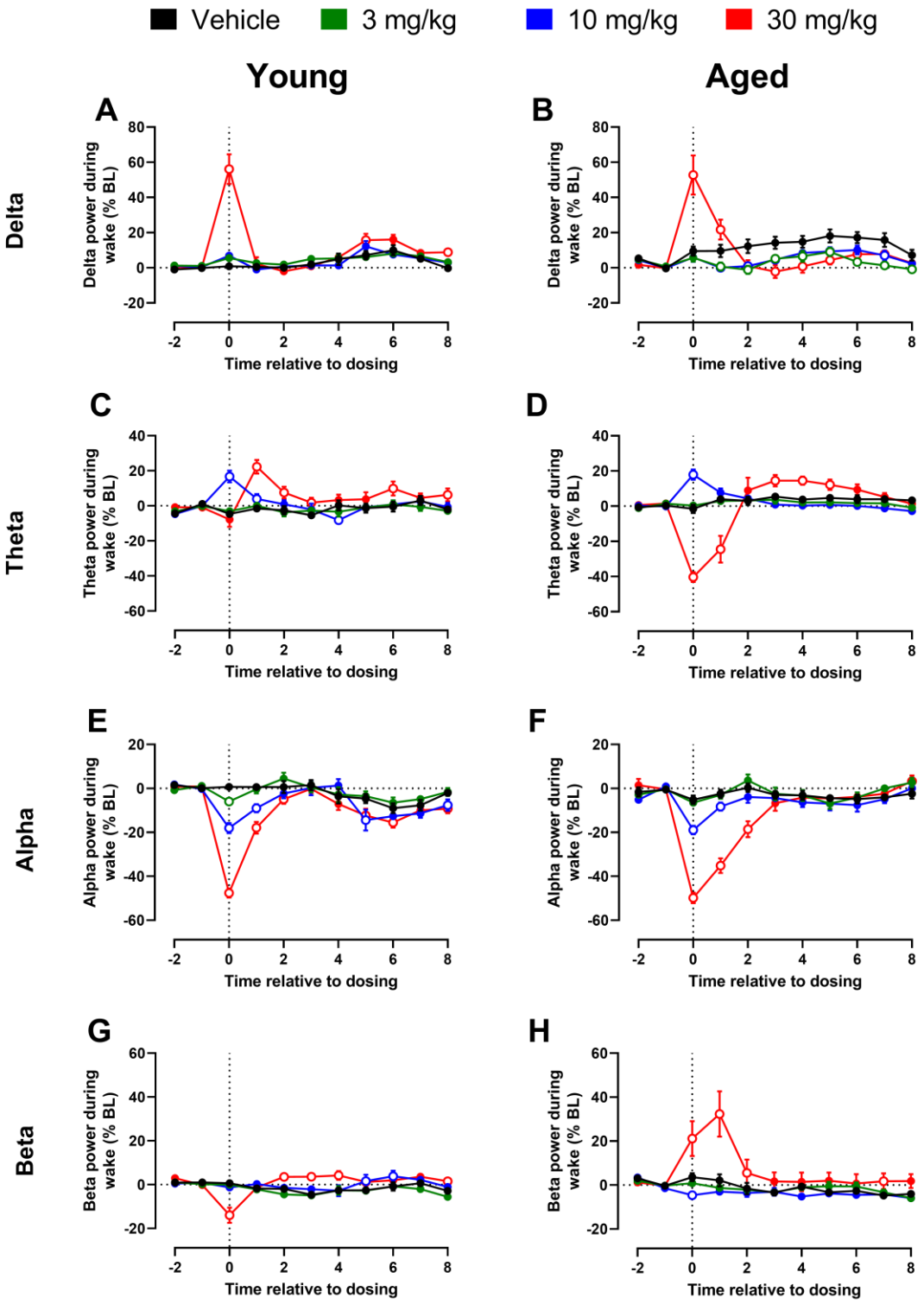

Figure 2.11. Time dependent effects of xanomeline on spectral power bands during wake in the active phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B), theta (C, D), alpha (E, F), and beta (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during wake epochs in the active phase following xanomeline dosing at time 0. Xanomeline produced initial dose related increases in delta power in young and aged mice with reduced delta power seen in aged mice 1-2 hours after dosing (A and B). Xanomeline produced dose related increases in theta power after dosing in young mice (C), in aged mice increased theta power was observed at 10 mg/kg, at 30 mg/kg an initial decrease followed by increased theta power was observed (D). Xanomeline produced a dose dependent decease in alpha power in young and aged mice (E and F). In young mice xanomeline produced a dose related decrease in beta power, followed by an increase (G), in aged mice an increase in beta power was observed at 30 mg/kg xanomeline (H). Data are expressed as means ± S.E.M. in 1-hour bins, n=14/group, open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

2.10C), with general shifts to lower powers, including reductions in beta and alpha powers and increases in theta and delta power across time (Figure 2.11). During NREM sleep following dosing with the 30 mg/kg dose of xanomeline, young mice displayed reductions in delta, alpha, and beta power and increased theta and gamma power (dose, time, and dose x time interaction, all $p<0.0001$) (Figure 2.10B). Across time, delta power (SWA) during NREM sleep was decreased after administration with the 10 and 30 mg/kg doses of xanomeline; following a transient increase with 30 mg/kg dose of xanomeline (dose, $p=0.4110$; time and dose x time, $p<0.0001$) (Figure 2.10D). Consistent with this reduction in delta power (SWA), increases in theta, gamma, and beta power at the 10 mg/kg dose were observed in the young mice, with reductions in alpha and beta power, and increased gamma power following the 30 mg/kg dose of xanomeline (Figure 2.11).

In non-pathologically aged mice, xanomeline at the 30 mg/kg dose reduced alpha power and increased beta and gamma power consistent with increased arousal, with more modest increases in gamma power observed with the 10 mg/kg dose of xanomeline during wake (dose, $p=0.0004$; frequency and dose x frequency, both $p<0.0001$) (Figure 2.10E). As shown in Figure 2.10G, increases in gamma power were observed at all doses of xanomeline tested (dose, $p=0.0116$; time and dose x time, $p<0.0001$). In support of this, a shift to higher powers was observed at the 3 and 10 mg/kg dose of xanomeline with reductions at delta power at the 3 mg/kg dose, and in delta and alpha power at 10 mg/kg dose in the young mice. The 30 mg/kg dose of xanomeline shifted the frequencies from theta and alpha to beta and gamma, with a transient

increase in delta power observed (Figure 2.11). Overall xanomeline had no dose-related effect on spectral power during NREM sleep in the 1-2 hours following dosing (dose, p=0.8246; frequency

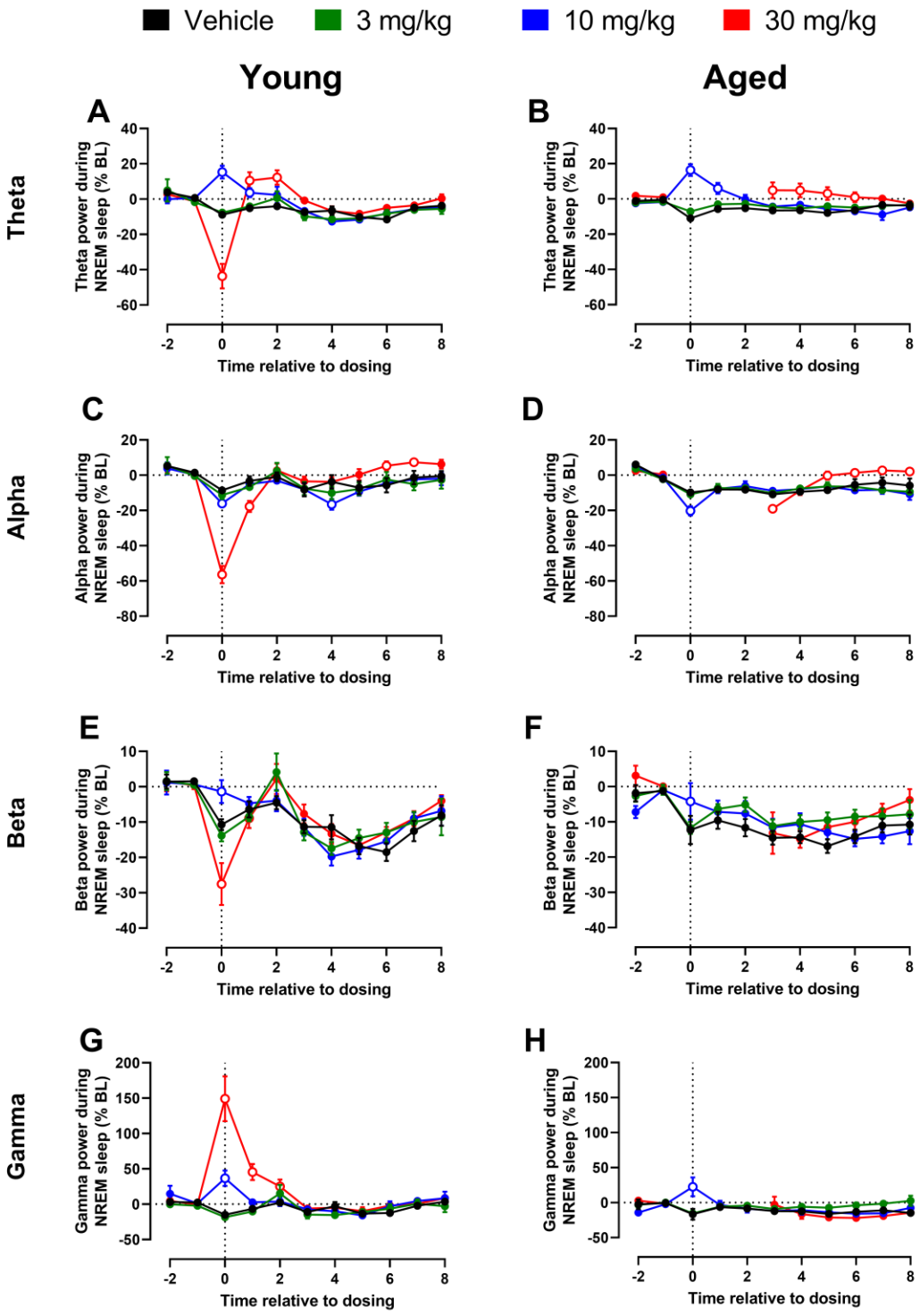

Figure 2.12. Time dependent effects of xanomeline on spectral power bands during NREM sleep in the active phase. Shown is the power relative the 1–2-hour baseline following light change within theta (A, B), alpha (C, D), beta (E, F), and gamma (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during NREM sleep epochs in the active phase following xanomeline dosing at time 0. Xanomeline produced a dose related increased theta power in young and aged mice, in young mice an initial reduction in theta power was seen following 30 mg/kg dosing (A, B). Xanomeline produced a dose related decrease in alpha power in young and aged mice, with a rebound increased alpha power seen 5-8-hrs after dosing (C, D). Xanomeline produced no consistent dose related effect on beta power during NREM sleep in young and aged mice (E and F). Xanomeline produced a dose related increase in gamma power in young and aged mice following dosing (G and H). Data are expressed as means ± S.E.M. in 1-hour bins, n=14/group, all time points in time courses contain n=5-14 mice. Time points with fewer than 14 are due to not all mice displaying NREM sleep. Open symbols indicate $p<0.05$ compared to vehicle (RM mixed effect model matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

$p<0.0001$; dose x frequency, $p=0.4447$) (Figure 2.10F). 10 mg/kg dose of xanomeline produced a transient decrease in delta power (SWA) during NREM sleep over time. The 30 mg/kg dose of xanomeline increased wakefulness in the 2 hours following dosing such that there were insufficient mice displaying NREM sleep to be analyzed (from -2 to 2 hr post dose: dose, $p=0.031$; time, $p=0.0448$ and dose x time, $p<0.0001$) (Figure 2.10H). In support of this decrease in delta power (SWA) during NREM, a shift to increased theta power was also observed with xanomeline in the non-pathologically aged mice, with additional shifts from alpha to beta and gamma powers (Figure 2.12).

In contrast, donepezil produced modest effects on gamma power, a correlate of arousal, during the active phase in young mice. The 3 mg/kg dose of donepezil increased delta, beta and lower gamma power and reduced alpha power (dose, $p<0.0001$; frequency, $p=0.0483$; dose x frequency, $p<0.0001$) (Figure 2.13A), with no consistent dose-related effect on total gamma power during wake (dose, $p=0.0744$; time, $p<0.0001$; dose x time, $p=0.0035$) in the young mice (Figure 2.13C). In support of these observations, donepezil produced time-dependent increases in delta power and shifts from alpha to beta power were observed (Figure 2.14). During NREM sleep, the 3 mg/kg dose of donepezil produced a small increase in delta power in the young mice (dose, $p=0.7494$; frequency, $p<0.0001$; dose x frequency, $p=0.0260$) (Figure 2.13B), however no dose-related effect on delta power (SWA) during NREM sleep was observed with the post hoc

tests (dose, p=0.4884; time, p<0.0001; dose x time interaction p=0.0035) (Figure 2.13D). Overall, the effects of donepezil on spectral powerbands across time were modest and transient. Beta and gamma powers were reduced following administration with the 0.1 and 0.3 mg/kg doses of

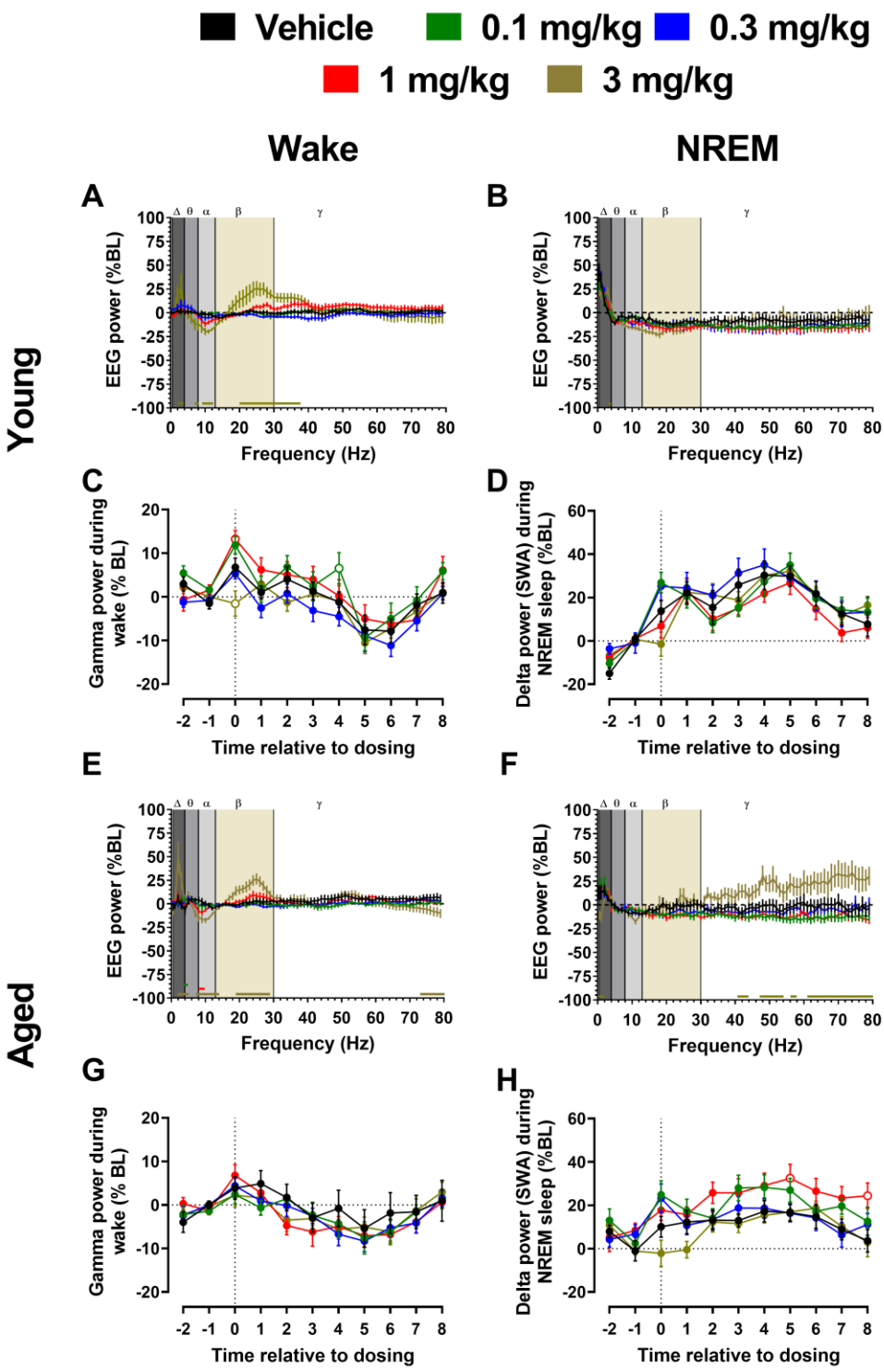

Figure 2.13. In the active phase, during wake donepezil had no effect on arousal in young and non-pathologically aged mice and produced shifts to higher powers during NREM sleep in non-pathologically aged mice. Shown is the relative spectral power during wake (A, E) and NREM sleep (B, F) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline, gamma power during wake (C, G) and SWA (relative delta power) during NREM sleep (D, H), during the active phase in young (A-D) and non-pathologically aged (E-H) mice. In young mice, during wake epochs 3 mg/kg donepezil increased delta, beta and gamma power, and reduced alpha power (A). Donepezil produced no dose related effect on spectral power during NREM sleep (B), with inconsistent effects observed on gamma power during wake across the active phase (C) and no effect on delta power (SWA) during NREM sleep across the active phase (D). In non-pathologically aged mice 3 mg/kg donepezil increased delta and beta power and reduced alpha and gamma power during wake epochs (E). During NREM sleep donepezil increased gamma power (F). No significant change in gamma power during wake across the active phase (G) or delta power (SWA) during NREM sleep around dosing time was observed, with a modest increase in delta power (SWA) 5 and 8 hours after dosing with 1 mg/kg donepezil (H). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A, B, E, F) and means ± S.E.M. in 1-hour bins. (C, D, G, H), n=12-14/group, all time points in time courses contain n=10-14 mice (C, D, G, H). Groups with fewer than 14 are due to not all mice displaying NREM sleep. Solid bars indicate $p<0.05$ compared to vehicle (A, B, E, F), open symbols indicate $p<0.05$ compared to vehicle (C, D, G, H) (RM 2-way ANOVA matching by both factors followed by Dunnett's test for A, C, E, G and RM mixed effect model matching by both factors followed by Dunnett's test for B, D, F, H). See table 2.4 for full statistical analysis.

donepezil, while increased gamma power and reduced alpha power were seen with the 3 mg/kg dose of donepezil in the young mice (Figure 2.15).

In the non-pathologically aged mice, during wake epochs the 3 mg/kg dose of donepezil increased delta and beta power and reduced alpha and gamma power (dose, $p<0.4513$; frequency and dose x frequency, $p<0.0001$) (Figure 2.13E), with no effect on total gamma power during wake over time (dose, $p=0.9580$; time $p<0.0001$; dose x time $p=0.6851$) (Figure 2.13G). Similar to young mice, donepezil increased delta power with a shift from alpha to theta power and decreased theta power in the non-pathologically aged mice (Figure 2.14). During NREM sleep, the 3 mg/kg dose of donepezil reduced delta power and increased gamma power in the non-pathologically aged mice (dose, $p=0.0013$, frequency and dose x frequency, both $p<0.0001$) (Figure 2.13F), with modest increases in delta power (SWA) during NREM sleep observed 5- and 8-hours following administration with the 1 mg/kg dose of donepezil (dose, $p=0.0316$; time and dose x time interaction, both $p<0.0001$) (Figure 2.13H). Additionally, alpha power was increased with the 0.1, 0.3, and 1 mg/kg doses of donepezil in the non-pathologically aged mice, while

gamma power was reduced with the 0.1 mg/kg dose of donepezil and increased with the 3 mg/kg dose of donepezil (Figure 2.15).

Xanomeline and donepezil reduced NREM sleep quality when dosed in the inactive phase.

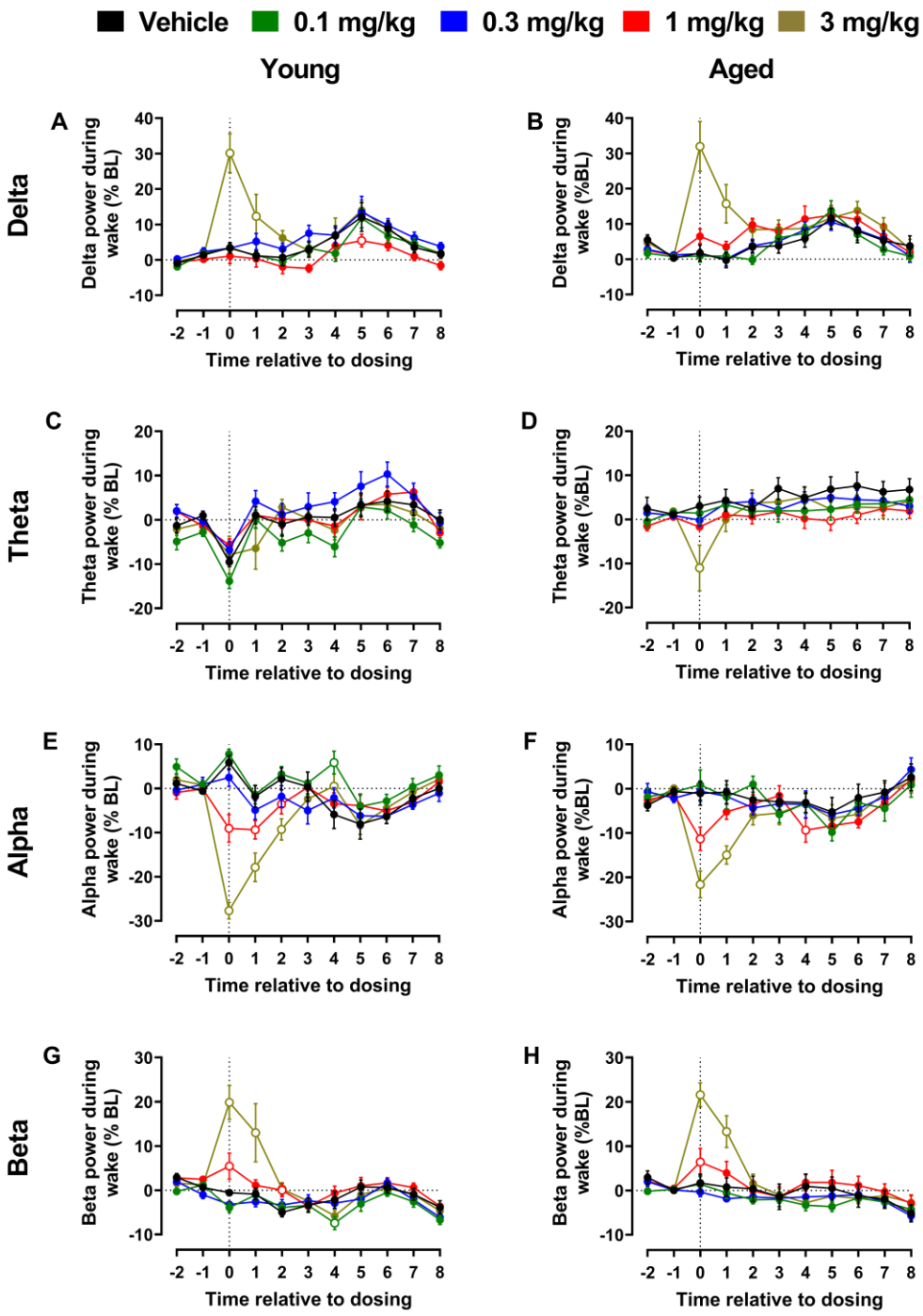

Figure 2.14. Time dependent effects of donepezil on spectral power bands during wake in the active phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B), theta (C, D), alpha (E, F), and beta (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during wake epochs in the active phase following donepezil dosing at time 0. Donepezil produced dose related increased delta power in young and aged mice (A, B), a transient reduction in theta power only in aged mice (C, D), a dose related reduction in alpha power in young and aged mice (E, F) and a dose related increase in beta power in young and aged mice (G and H). Data are expressed as means ± S.E.M. in 1-hour bins, n=14/group, open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

Given the wake and arousal promoting effects observed with xanomeline, we next assessed the effects of xanomeline and donepezil on relative spectral power when dosed in the inactive phase in both the young and non-pathologically aged mice. In the young mice, xanomeline dose-dependently increased gamma power and reduced delta power during wake. Additionally, the 30 mg/kg dose of xanomeline increased theta power (dose, $p=0.0211$; frequency and dose x frequency, both $p<0.0001$) (Figure 2.16A). Increased total gamma power during wake across time following the 30 mg/kg dose of xanomeline was observed, with transient reductions observed at the 3 and 10 mg/kg doses of xanomeline (dose, $p<0.0266$; time and dose x time, both $p<0.0001$) (Figure 2.16D). Consistent with this shift to higher frequencies, reductions in delta power were also observed with xanomeline in the young mice. Transient increases in theta power were noted at the 3 and 10 mg/kg doses of xanomeline with a decrease in theta power following the 30 mg/kg dose of xanomeline. Alpha and beta power were reduced following dosing at the 10 and 30 mg/kg doses of xanomeline with increased alpha power observed with the 3 mg/kg dose of xanomeline in the young mice (Figure 2.17). During NREM sleep, all doses of xanomeline produced reductions in delta power; the 30 mg/kg dose of xanomeline also decreased alpha power and increased theta and gamma power (dose, frequency and dose x frequency interaction, all $p<0.0001$) (Figure 2.16B). The dose-dependent reductions in delta power (SWA) produced by xanomeline were observed across time during NREM sleep in the young mice (-2 to 1-hr post dose: dose, $p=0.0163$; time and dose x time interaction, both $p<0.0001$; 2 to 8-hr post dose: dose,

p=0.0293; time and dose x time interaction, both p<0.0001) (Figure 2.16E). Consistent with the xanomeline induced decreases in delta power (SWA), a shift to theta power was also seen with

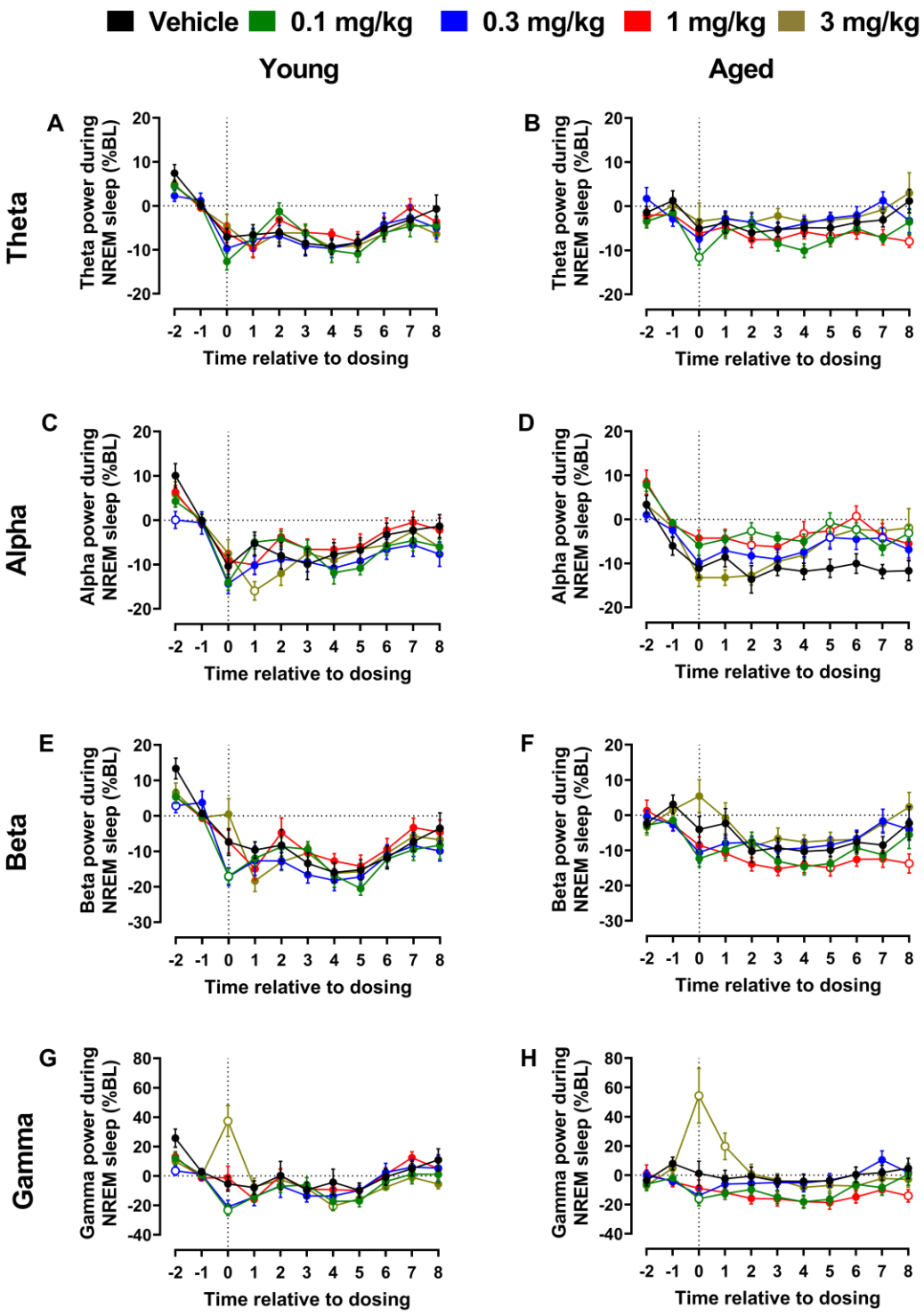

Figure 2.15. Time dependent effects of donepezil on spectral power bands during NREM sleep in the active phase. Shown is the power relative the 1–2-hour baseline following light change within theta (A, B), alpha (C, D), beta (E, F), and gamma (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during NREM sleep epochs in the active phase following donepezil dosing at time 0. Donepezil produced no consistent dose related effect on theta power in young or aged mice (A, B), a transient decrease in alpha power at the highest dose in young mice (C) and a delayed increased alpha power at all doses tested (D). No consistent dose related effect was observed in beta power (E and F), and a dose related increase in gamma power at the highest dose, and a reduction in gamma power at lower doses in young and aged mice (G, H). Data are expressed as means ± S.E.M. in 1-hour bins, n=14/group, all time points in time courses contain n=10-14 mice. Time points with fewer than 14 are due to not all mice displaying NREM sleep. Open symbols indicate p<0.05 compared to vehicle (RM mixed effect model matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

decreases alpha and increases beta and gamma power in the young mice (Figure 2.18). During REM sleep, the 10 mg/kg dose of xanomeline reduced delta power in the young mice (dose, p=0.8089; frequency and dose x frequency interaction, both p<0.0001) (Figure 2.16C).

In non-pathologically aged mice during wake, xanomeline reduced alpha power and increased beta and gamma power, consistent with increased arousal (dose, frequency and dose x frequency interaction, all p<0.0001) (Figure 2.16F). Xanomeline produced dose-dependent increases gamma power across time (dose, p=0.0049; time and dose x time, both p<0.0001) (Figure 2.16I). Consistent with this shift to higher powers, reduced delta frequency was seen following a transient increase after administration of the 30 mg/kg dose of xanomeline, along with reduced theta and alpha power and increased beta power in the non-pathologically aged mice (Figure 2.17). During NREM sleep, the 3 and 10 mg/kg doses of xanomeline reduced delta power, while the 10 mg/kg dose of xanomeline decreased alpha power and increased gamma power in the non-pathologically aged mice (dose, p=0.2131; frequency and dose x frequency interaction, both p<0.0001) (Figure 2.16G). Xanomeline produced a dose-dependent reduction in delta power (SWA) during NREM sleep across time in the non-pathologically aged mice with significance at all doses (-2 to 1-hr post dose: dose, time and dose x time interaction, all p<0.0001; 2 to 8-hr post dose: dose, p=0.0080; time and dose x time, both p<0.0001) (Figure 2.16J). Similar to young mice, increased theta power, decreased alpha, and increased gamma power were observed in the non-pathologically aged mice (Figure 2.18). During REM sleep, the 10 mg/kg dose of

xanomeline decreased delta power and increased alpha power in the non-pathologically aged mice (dose, p=0.9999; frequency, p<0.0001; dose x frequency p=0.0020) (Figure 2.16H).

Donepezil also produced disruptions during NREM sleep when dosed in the inactive phase. In the young mice during wake, the 1 and 3 mg/kg doses of donepezil decreased delta power and increased alpha and gamma power, consistent with increased arousal (dose, p=0.0002; frequency and dose x frequency, both p<0.0001) (Figure 2.19A). Donepezil increased

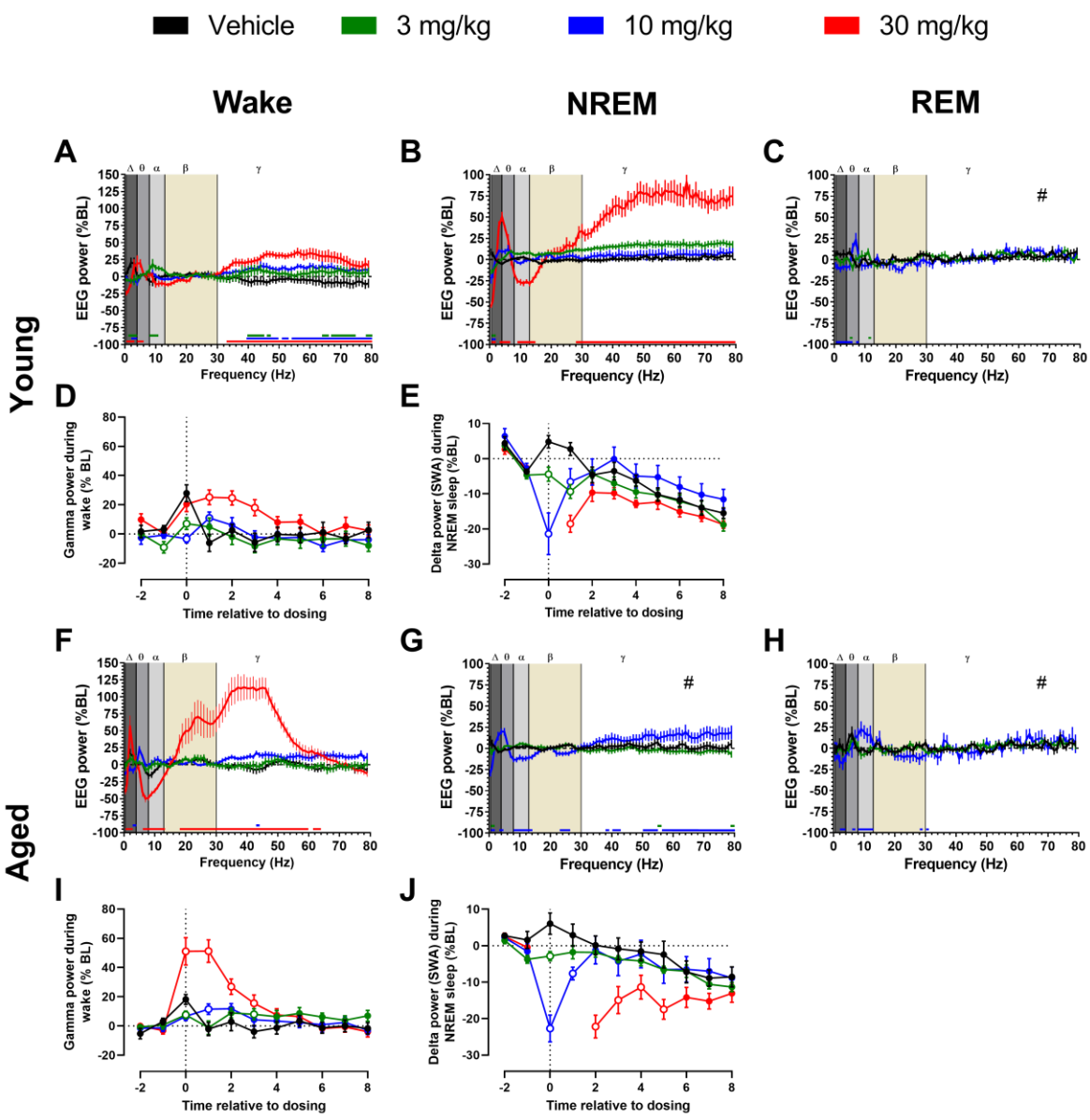

Figure 2.16. During the inactive phase, xanomeline increased arousal during wake and reduced delta power (SWA) in NREM sleep in young and non-pathologically aged mice. Shown is the relative spectral power during wake (A, F), NREM sleep (B, G) and REM sleep (C, H) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline, gamma during wake (D, I) and relative delta power (SWA) during NREM sleep (E, J), following xanomeline dosing during the inactive phase in young (A-E) and non-pathologically aged (F-J) mice. In young mice, during wake epochs, 3, 10 and 30 mg/kg xanomeline decreased delta power and increased gamma power. 3mg/kg increased alpha power, and 30 mg/kg increased theta power in the 1-2 hours following dosing (A). During NREM sleep, all doses decreased delta power and 30 mg/kg additionally increased theta power, reduced alpha power and increased gamma power in the 1-2 hours following dosing (B). During REM sleep 10 mg/kg xanomeline decreased delta power and increased theta power, insufficient mice displayed REM sleep in the 30 mg/kg xanomeline group in the 1-2 hours following dosing (C). Gamma power during wake was dose dependently increased following 10 and 30 mg/kg xanomeline (D) and delta power (SWA) during NREM sleep displayed a dose dependent decrease following all doses of xanomeline (E). In non-pathologically aged animals, during wake epochs 30 mg/kg xanomeline produced decreased theta and alpha power and increased beta and gamma power in the 1-2 hours following dosing (F). During NREM sleep 10 mg/kg xanomeline decreased delta, alpha and beta power, and increased theta and gamma power, insufficient mice displayed NREM sleep in the 30 mg/kg xanomeline group in the 1-2 hours following dosing (G). During REM sleep 10 mg/kg xanomeline decreased delta power and increased alpha power, insufficient mice displayed REM sleep in the 30 mg/kg xanomeline group in the 1-2 hours following dosing (H). Gamma power during wake was increased following dosing with 10 and 30 mg/kg xanomeline (I), with a dose dependent decrease in delta power (SWA) during NREM observed (J). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A-C, F-H) and means ± S.E.M. in 1-hour bins (D, E, I, J), n=7-14/group, all time points in time courses contain n=7-14 mice (D, E, I, J). Groups with fewer than 13-14 are due to not all mice displaying NREM or REM sleep, # indicates fewer than 5 mice display NREM or REM sleep in the 30 mg/kg dose group, so this was excluded. Solid bars indicate $p<0.05$ compared to vehicle (A, B, C, F, G, H), open symbols indicate $p<0.05$ compared to vehicle (D, E, I, J), ** indicates main effect of dose $p<0.01$ (J) (RM 2-way ANOVA matching by both factors followed by Dunnett's test for A, D, F, I and RM mixed effect model matching by both factors followed by Dunnett's test for B, C, E, G, H, J). See table 2.4 for full statistical analysis.

gamma power across time during wake with the 1 and 3 mg/kg doses in the young mice (dose, $p=0.0739$; time, $p<0.0001$; dose x time, $p=0.0011$) (Figure 2.19D). Consistent with this shift to higher powers, reductions in delta and theta power were seen, with transient increases in theta power following administration of the 3 mg/kg dose of donepezil (Figure 2.20).

During NREM sleep, the 1 mg/kg dose of donepezil produced modestly increased gamma power and the 3 mg/kg dose of donepezil modestly decreased delta and increased theta power in the young mice (dose, $p=0.0290$, frequency and dose x frequency, both $p<0.0001$) (Figure 2.19B). The decreased delta power (SWA) during NREM sleep observed with the 3 mg/kg dose of donepezil was followed by a small rebound (dose, $p=0.5619$; time and dose x time, both $p<0.0001$) (Figure 2.19E). Consistent with this shift away from delta power, increased theta, beta, and gamma powers were observed with donepezil, with rebound reductions in theta, alpha, and beta power in the young mice (Figure 2.21). During REM sleep, there were no dose-related effects

produced by donepezil in the young mice (dose, p=0.8469; frequency, p=0.0082 and dose x frequency, p>0.9999) (Figure 2.19C).

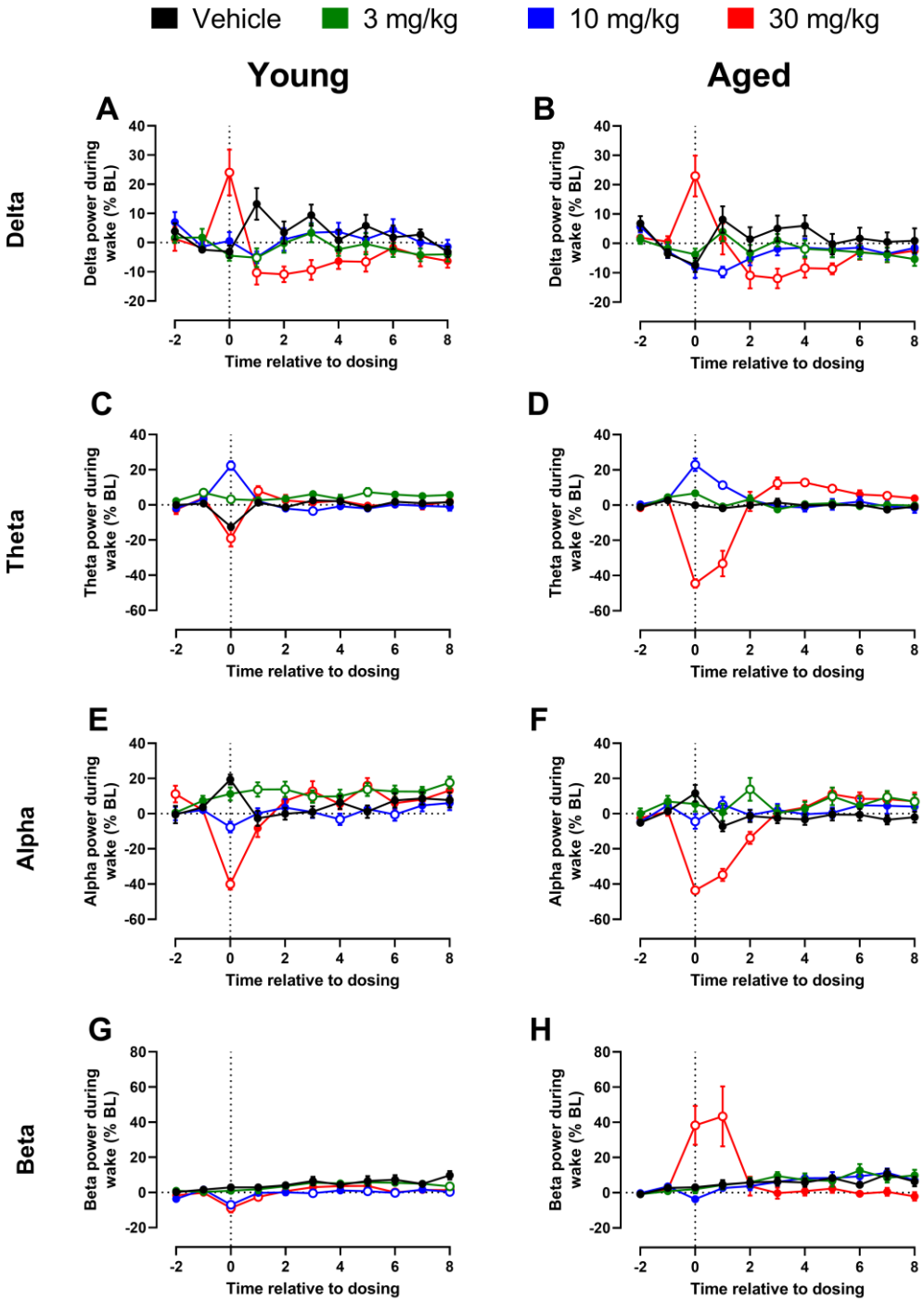

Figure 2.17. Time dependent effects of xanomeline on spectral power bands during wake in the inactive phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B), theta (C, D), alpha (E, F), and beta (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during wake epochs in the inactive phase following xanomeline dosing at time 0. Xanomeline produced a dose related increase in delta power followed by decreased delta power in young and aged mice (A, B). The 30 mg/kg dose of xanomeline decreased theta power followed by a later increase in young and old mice, whereas 10 mg/kg xanomeline increased theta power (C, D). Xanomeline produced dose related decreased alpha power in young and aged mice (E, F). In young mice xanomeline produced a modest decrease in beta power (G), in aged mice increased beta power was observed (H). Data are expressed as means ± S.E.M. in 1-hour bins, n=13-14/group, open symbols indicate p<0.05 compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

During wake, the non-pathologically aged mice displayed decreased theta and increased beta and gamma power following the 3 mg/kg dose of donepezil (dose, p=0.0007; frequency and dose x frequency, both p<0.0001) (Figure 2.19F). Increased gamma power across time during wake was also observed following the 3 mg/kg dose of donepezil (dose, p=0.0917; time and dose x time, both p<0.0001) (Figure 2.19I). In support of this shift to higher powers, the 3 mg/kg dose of donepezil produced decreased theta and alpha powers with increased beta power, as well as a transient modest increase in delta power in the non-pathologically aged mice (Figure 2.20). When assessing relative spectral power during NREM sleep, the 3 mg/kg dose of donepezil reduced delta power and increased beta and gamma powers in the non-pathologically aged mice (dose, frequency, and dose x frequency, all p<0.0001) (Figure 2.19G). This resulted in decreased delta power (SWA) during NREM sleep across time following the 3 mg/kg dose of donepezil (2 to 8-hrs post dose: dose, p=0.0399; time and dose x time, both p<0.0001) (Figure 2.19J). Similar to young mice, the non-pathologically aged mice display increased theta, beta, and gamma power, with reduced alpha power after donepezil treatment (Figure 2.21). Donepezil produced no dose-related effect on spectral power during REM sleep in the non-pathologically aged mice (dose, p=0.9466; frequency, p<0.0015; dose x frequency, p=0.4598) (Figure 2.19H).

Xanomeline and donepezil produced cholinergic adverse effects at higher doses in non-pathologically aged mice.

We assessed whether xanomeline and donepezil produced adverse side effects associated with the activation of peripheral M_2 and M_3 mAChRs in non-pathologically aged mice during the inactive and active phases, at doses that produced increased wakefulness and enhanced qEEG correlates of arousal, using the Modified Irwin neurological test battery (Table 2.5 and 2.6). During the active phase, the 30 mg/kg dose of xanomeline and the 3 mg/kg dose of

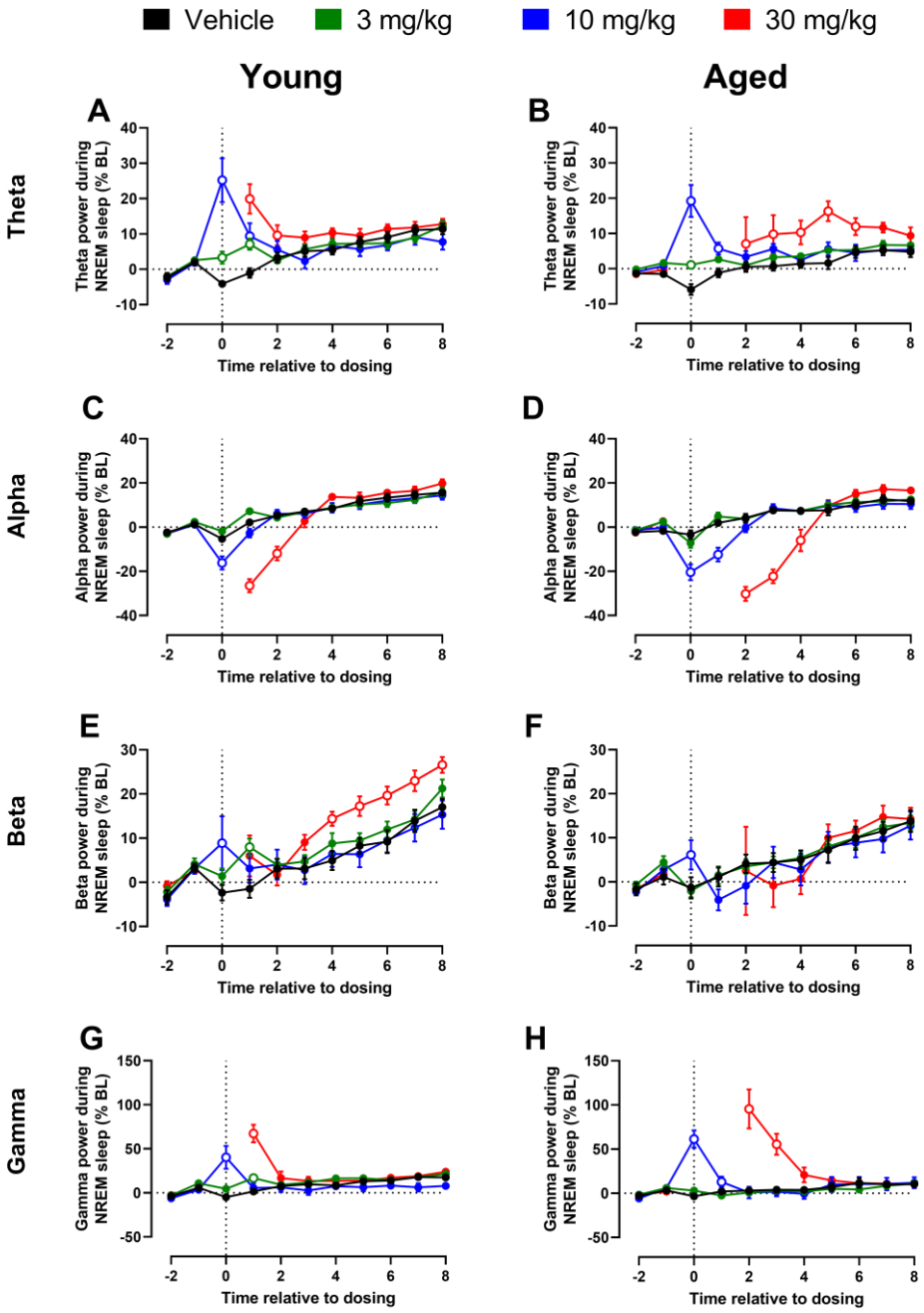

Figure 2.18. Time dependent effects of xanomeline on spectral power bands during NREM sleep in the inactive phase. Shown is the power relative the 1–2-hour baseline following light change within theta (A, B), alpha (C, D), beta (E, F), and gamma (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during NREM sleep epochs in the inactive phase following xanomeline dosing at time 0. Xanomeline produced dose related increased theta power in young and aged mice (A, B) and reduced alpha power (C, D). 10 mg/kg xanomeline produced a transient increase in beta power in young and aged mice, while 30 mg/kg produced a delayed increased beta power in young mice (E and F). Xanomeline produced dose related increased gamma power in young and aged mice (G, H). Data are expressed as means ± S.E.M. in 1-hour bins, n=13-14/group, all time points in time courses contain n=7-14 mice. Time points with fewer than 13-14 are due to not all mice displaying NREM sleep. Open symbols indicate p<0.05 compared to vehicle (RM mixed effect model matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

donepezil produced significant adverse effects consistent with the activation of peripheral M_2 and M_3 mAChRs when compared to the vehicle treated mice; and the 30 mg/kg dose of xanomeline induced greater adverse effects compared to the 3 mg/kg dose of donepezil (main effect of dose, $p<0.0001$; and time, $p=0.0002$; xanomeline vs vehicle, $p<0.0001$; donepezil vs vehicle: dose, $p=0.0238$; and xanomeline vs donepezil, $p=0.0003$) (Table 2.5). Similarly, during the inactive phase, significant adverse effects consistent with the activation of peripheral M_2 and M_3 mAChRs were observed following administration of the 30 mg/kg dose of xanomeline and the 3 mg/kg dose of donepezil compared to vehicle conditions; however no difference between the xanomeline- and donepezil-treated mice was observed (main effect of dose and time, $p<0.0001$; xanomeline vs vehicle, $p<0.0001$; donepezil vs vehicle: dose, $p<0.0001$; and no effect of xanomeline vs donepezil) (Table 2.6).

2.4. Discussion

Non-pathologically aged mice displayed multiple disruptions in sleep/wake architecture and arousal across the circadian rhythm. During the active phase, aged mice showed increased fragmentation of wake, as denoted by increased numbers of wake bouts and reduced wake bout durations. This observed fragmentation in wake in non-pathological aging was consistent with previous rodent studies (S. bin Li et al., 2022) and analogous to increased daytime napping observed in aging and AD clinical populations (S. Li et al., 2022; Spira et al., 2018). Furthermore,

this is the first study demonstrating that non-pathological aging in mice produces significant deficits in arousal during the active, but not inactive phase, characterized by decreased gamma power during wake epochs. Similar changes in arousal have been reported in clinical literature, where AD populations display shifts in spectral power from high to low frequency during wake, and in non-pathological aging, where decreased gamma power during wake is seen (D'Atri et al., 2021; Murty et al., 2020). In the inactive phase, aged mice showed only modest decreases in total

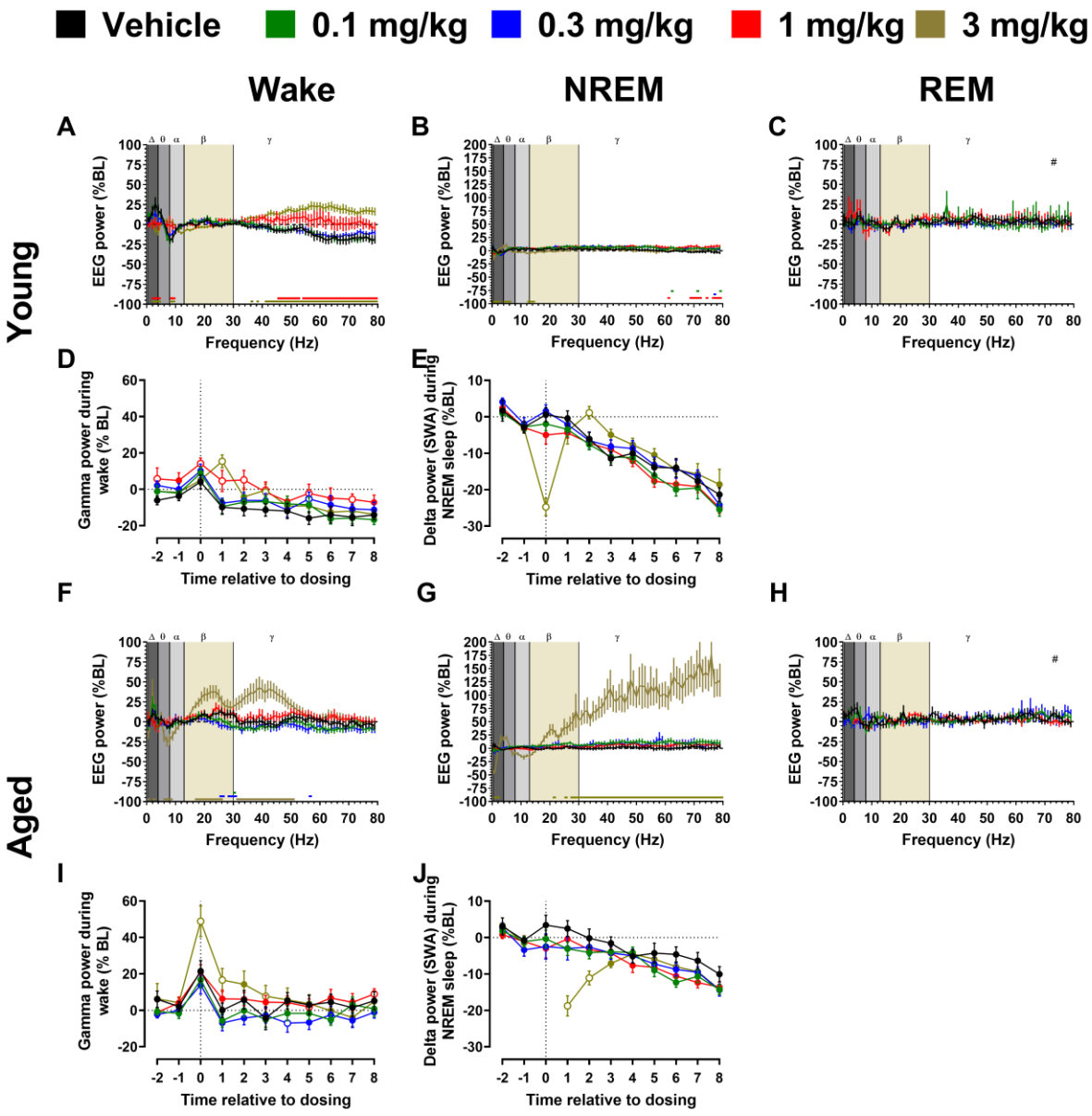

Figure 2.19. In the inactive phase, donepezil decreased delta power (SWA) during NREM and increased arousal wake in young and non-pathologically aged mice. Shown is the relative spectral power during wake (A, F), NREM sleep (B, G) and REM sleep (C, H) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline, gamma during wake (D, I) and relative delta power (SWA) during NREM sleep (E, J), following xanomeline dosing during the inactive phase in young (A-E) and non-pathologically aged (F-J) mice. In young mice, during wake 1 and 3 mg/kg donepezil decreased delta power and increased alpha power and gamma power in the 1-2 hours following dosing (A). During NREM sleep 1 mg/kg donepezil produced increased gamma power, while 3 mg/kg produced a modest increase in theta power and decreased beta power in the 1-2 hours following dosing (B). No dose related effects on spectral power during REM sleep were observed in the 1-2 hours following dosing, in the 3 mg/kg donepezil dose insufficient mice displayed REM sleep so this was excluded (C). Donepezil produced increased gamma power with effects seen at 1 and 3 mg/kg (D) and produced a transient decrease in delta power (SWA) during NREM sleep followed by a rebound increase at 3 mg/kg (E). In non-pathologically aged mice, during wake epochs, 3 mg/kg donepezil reduced theta power and increased beta and gamma power in the 1-2 hours following dosing (F). During NREM sleep 3 mg/kg donepezil reduced delta power and increased beta and gamma power in the 1-2 hours following dosing (G). No dose related effect on REM relative spectral was observed in the 1-2 hours following dosing, the 3 mg/kg donepezil dose had insufficient mice displaying REM sleep so was excluded (H). Donepezil increased gamma power following dosing in the 3 mg/kg group (I). 3 mg/kg donepezil produced reduced delta power (SWA) during NREM sleep (L). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A-C, F-H) and means ± S.E.M. in 1-hour bins (D, E, I, J), n=9-14/group, all time points in time courses contain n=10-14 mice (D, E, I, J). Groups with fewer than 13-14 are due to not all mice displaying NREM or REM sleep, # indicates fewer than 5 mice display NREM or REM sleep in the 3 mg/kg dose group so this was excluded. Solid bars indicate $p<0.05$ compared to vehicle (A, B, C, F, G, H), open symbols indicate $p<0.05$ compared to vehicle (D, E, I, J) (RM 2-way ANOVA matching by both factors followed by Dunnett's test for A, D, F, I and RM mixed effect model matching by both factors followed by Dunnett's test for B, C, E, G, H, J). See table 2.4 for full statistical analysis.

NREM sleep duration, with no observed change in wake or arousal. Overall, the impact of aging in mice resulted in circadian-dependent changes in sleep/wake architecture and arousal highlighting the importance in future studies of evaluating preclinical AD disease models alone or in combination with novel pharmacological challenges across the diurnal rhythm.

Numerous studies have explored the changes in cholinergic signaling associated with circadian rhythm and/or aging (Bartus et al., 1982; Dumas and Newhouse, 2011; Mitsushima et al., 1996). Previous studies have demonstrated that central ACh levels are highest during the active phase and lowest in the inactive phase in rodents (Mitsushima et al., 1996). With increasing age, cholinergic signaling in rodents stops displaying its normal circadian changes (Mitsushima et al., 1996), which may explain the more profound wake fragmentation and arousal deficits observed in the present study in the active phase of non-pathologically aged mice. Changes in cholinergic function during non-pathological aging in mice may also explain the observed differences in the efficacy of indirect- and direct-acting muscarinic cholinergic agonists on

normalizing wake fragmentation and arousal deficits across the circadian cycle. Specifically, during the inactive phase when cholinergic signaling is low, donepezil and xanomeline increased wakefulness and arousal in the young mice. In the non-pathologically aged mice, donepezil and xanomeline increased wakefulness, with donepezil modestly increasing arousal, while

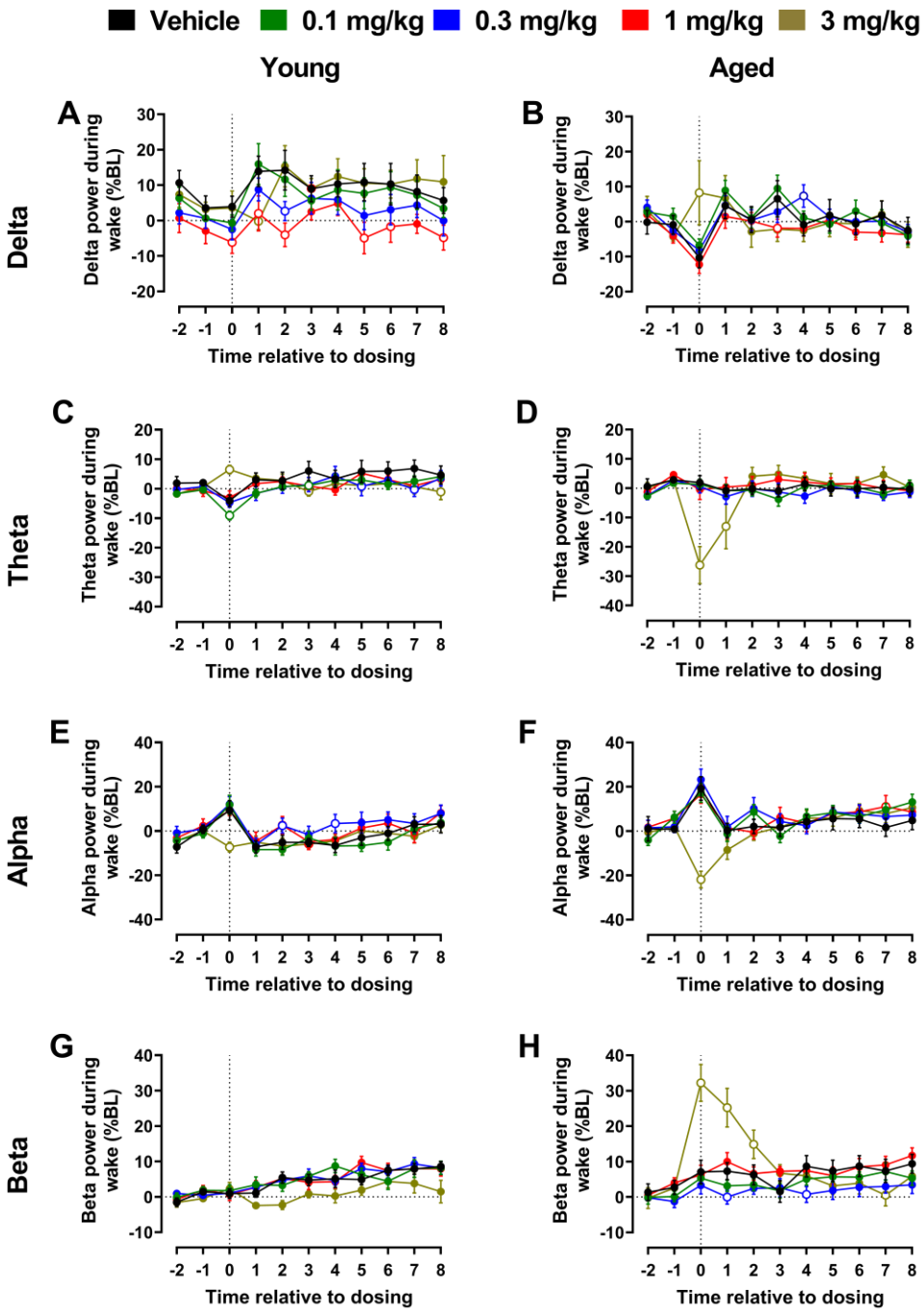

Figure 2.20. Time dependent effects of donepezil on spectral power bands during wake in the inactive phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B), theta (C, D), alpha (E, F), and beta (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during wake epochs in the inactive phase following donepezil dosing at time 0. Donepezil produced dose related decreased delta power in young mice with no consistent effect observed in aged mice (A, B). Donepezil produced transient changes in theta power in young mice, while in aged mice a robust reduction in theta power was observed (C, D). Donepezil reduced alpha power in young and aged mice (E, F), and increased alpha power in aged mice with no effect seen in young mice (G, H). Data are expressed as means ± S.E.M. in 1-hour bins, n=13-14/group, open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

xanomeline robustly enhanced arousal. In contrast, during the active phase in the young mice when cholinergic signaling is high, donepezil produced no effect on wake and arousal, whereas xanomeline produced an increase in wake with no effect on arousal. During the active phase in the non-pathologically aged mice, donepezil again had no effect on wake or arousal, while xanomeline produced marked increases in both wakefulness and arousal. We previously demonstrated that young rodents display reduced arousal during wake in the inactive phase (Gould et al., 2016). In light of these and the present findings, we hypothesize that young rodents in the active phase exhibit optimal arousal associated with high levels of cholinergic signaling during wake, such that there is insufficient dynamic range in cholinergic tone to further boost arousal. However, the non-pathologically aged mice displayed a deficit in arousal during the active phase, which may be attributed to the previously described age-related reductions in cholinergic signaling. Such a deficit in cholinergic signaling during the active phase in non-pathologically aged mice suggests that boosting arousal may be possible through the direct activation of M_1 and/or M_4 mAChRs using the direct-acting muscarinic cholinergic agonist xanomeline. While in contrast, boosting diminished cholinergic signaling with the acetylcholinesterase inhibitor donepezil may not provide sufficient enhancement of central cholinergic signaling at cortical M_1 and/or M_4 mAChR subtypes to observe improvements in arousal. Ongoing studies are evaluating the integrity of cholinergic basal forebrain projections, signaling and muscarinic receptor density in non-pathologically aged mice to confirm this hypothesis.

The present findings support further development of pharmacologic approaches, such as direct-acting muscarinic cholinergic agonists like xanomeline, to boost cholinergic signaling at M_1 and M_4 mAChRs in aging, MCI and AD. Historical *in vitro* studies suggested that the direct-acting

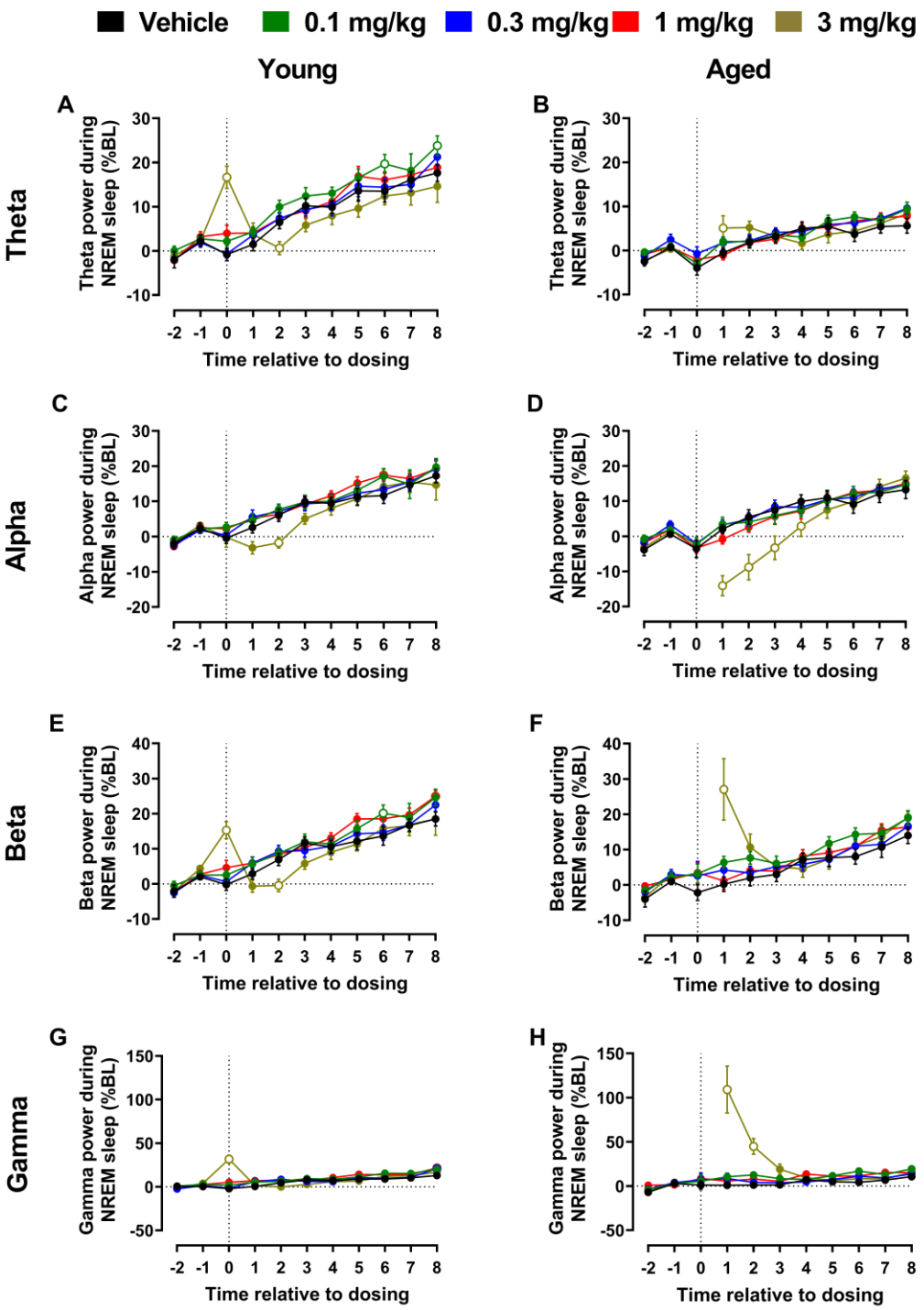

Figure 2.21. Time dependent effects of donepezil on spectral power bands during NREM sleep in the inactive phase. Shown is the power relative the 1–2-hour baseline following light change within theta (A, B), alpha (C, D), beta (E, F), and gamma (G, H) power bands in young (A, C, E, G) and non-pathologically aged (B, D, F, H) mice during NREM sleep epochs in the inactive phase following donepezil dosing at time 0. Donepezil produced dose related increased theta power in young and aged animals followed by transient decreased delta in young mice (A, B). Donepezil produced dose related increased alpha power in young and aged animals (C, D). Increased beta was observed following donepezil dosing in young and aged mice, with a later decrease observed in young mice (E, F). Donepezil produced dose related increased gamma power in young and aged mice (G, H). Data are expressed as means ± S.E.M. in 1-hour bins, n=13-14/group, all time points in time courses contain n=10-14 mice. Time points with fewer than 13-14 are due to not all mice displaying NREM sleep. Open symbols indicate $p<0.05$ compared to vehicle (RM mixed effect model matching by both factors followed by Dunnett's test). See table 2.4 for full statistical analysis.

muscarinic cholinergic agonist xanomeline displays partial agonism preferentially at the M_1 and M_4 mAChRs (Bymaster et al., 1997), which was thought to contribute to its improved adverse effect profile and enhanced efficacy observed relative to AChEIs. However, the present *in vivo* data using the Modified Irwin neurological test battery do not support the idea of an improved adverse effect profile of xanomeline; we actually observed that xanomeline causes more pronounced adverse effects relative to donepezil. To achieve a broader therapeutic index, a formulation of xanomeline, with the peripherally restricted non-selective mAChR antagonist trospium, known as KarXT, has been developed, which is currently undergoing clinical trials(Brannan et al., 2021) (ClinicalTrials.gov: NCT03697252, NCT04659161, NCT05511363). With regards to the mechanism of action of xanomeline, recent *in vitro* pharmacology studies have demonstrated that xanomeline exhibits unique biased agonism activity, with significant bias away from ERK1/2 phosphorylation and Ca^{2+} mobilization signaling pathways compared to $G\alpha_{i2}$ activation, at the recombinant human M_4 mAChR subtype (McDonald et al., 2022), which may also account for its unique efficacy profile in preclinical and clinical studies.

As xanomeline activates the M_1 and M_4 mAChRs further studies will be needed to assess whether the enhancement in wake and arousal is primarily M_1 or M_4 mediated. Different approaches to provide greater M_1 and M_4 activation without producing dose-limiting adverse effects, seen both in clinical studies (Bender et al., 2017; Dunn et al., 2000; Rogers et al., 1998) and in the present data, include compounding the direct-acting muscarinic cholinergic agonist

xanomeline with the peripherally restricted muscarinic antagonist trospium (Brannan et al., 2021) (ClinicalTrials.gov: NCT03697252, NCT04659161, NCT05511363). Alternatively, allosteric ligands have been shown to display improved muscarinic selectivity compared to direct acting orthosteric agonists and may provide a different mechanism through which to achieve greater selectivity with improved M_1 or M_4 activation than seen with the indirect-acting AChEIs (Bubser et al., 2014; Ghoshal et al., 2016; Gould et al., 2020).

Sleep disruptions are a well-characterized symptom of AD, with recent work suggesting that sleep disruptions may also lead to increased AD pathology (C. Wang and Holtzman, 2020). Numerous studies have indicated that AChEIs, including donepezil, may lead to increased sleep disturbances (Hsieh et al., 2022). Our current data set supports this, with donepezil reducing NREM sleep quality in aged mice when dosed in the inactive phase. Xanomeline administered in the inactive phase produced a similar decrease in NREM sleep quality. This provides further evidence that the time in the circadian cycle when these compounds are administered is vitally important. One consideration with the present data is that the reported effects were observed following acute dosing. In clinical populations the donepezil dose is escalated over several weeks until a stable chronic maintenance dose is achieved (Winblad et al., 2001). The NREM sleep disruptions and peripheral side effects observed with donepezil and xanomeline may decrease with chronic dosing. Future studies will be needed to assess the effects of xanomeline on sleep/wake architecture following chronic dosing.

In conclusion, this study is the first to systematically assess circadian-dependent pharmacological effects of the direct-acting muscarinic cholinergic agonist xanomeline and indirect-acting muscarinic cholinergic agonist donepezil on sleep/wake architecture and spectral power in young and aged mice. The data presented here indicate that when considering treatment for a disease process that occurs during aging, it is of critical importance to understand the efficacy of the compound in the aging process across the circadian cycle. These findings support the future development of ligands like xanomeline that directly target M_1 and/or M_4 mAChRs

subtypes. Future studies in higher order species will be essential to test the hypothesis that direct-acting muscarinic cholinergic agonists provide improved symptomatic benefit if dosed during the day in MCI and AD populations compared to indirect-acting muscarinic cholinergic agonists such as the acetylcholinesterase inhibitor donepezil.

Figure	Age	Experiment	Measure	Phase	Statistical Test	Comparison	Degrees of freedom	F or *t*	p	*	Group Size	Post hoc results
1a	Comparison	Young vs aged time in wake	Duration (min/2hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 54	3.781	0.0571	ns	28	Young vs Aged: ZT0
						Time	11, 594	141.6	<0.0001	****		
						Age x Time	11, 594	2.966	0.0008	***		
1b	Comparison	Young vs aged time in NREM	Duration (min/2hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 54	2.640	0.1100	ns	28	Young vs Aged: ZT16 and 0
						Time	11, 594	124.7	<0.0001	****		
						Age x Time	11, 594	3.475	0.0001	***		
1c	Comparison	Young vs aged time in REM	Duration (min/2hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 54	5.238	0.0260	*	28	Young vs Aged: ZT18 and 20
						Time	11, 594	164.4	<0.0001	****		
						Age x Time	11, 594	3.731	<0.0001	****		
1d	Comparison	Young vs aged time in wake	Duration (min/12hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 54	3.781	0.0571	ns	28	N/A
						Time	1, 54	675.3	<0.0001	****		
						Age x Time	1, 54	3.781	0.0771	ns		
1e	Comparison	Young vs aged time in NREM	Duration (min/12hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 54	2.640	0.1100	ns	28	Young vs Aged: ZT0-ZT12
						Time	1, 54	559.1	<0.0001	****		
						Age x Time	1, 54	6.990	0.0107	*		
1f	Comparison	Young vs aged time in REM	Duration (min/12hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 54	5.238	0.0260	*	28	Young vs Aged: ZT12-ZT24
						Time	1, 54	950.8	<0.0001	****		
						Age x Time	1, 54	2.247	0.1397	ns		
2a	Comparison	Young vs aged wake bout number	Bout number (ZT14 - ZT22)	Inactive	Students t-test	Age	54	4.802	<0.0001	****	28	N/A
2b	Comparison	Young vs aged wake bout duration	Average bout duration (ZT14 -	Inactive	Students t-test	Age	54	3.308	0.0017	**	28	N/A
2c	Comparison	Young vs aged NREM bout number	Bout number (ZT14 - ZT22)	Inactive	Students t-test	Age	54	5.187	<0.0001	****	28	N/A
2d	Comparison	Young vs aged NREM bout duration	Average bout duration (ZT14 -	Inactive	Students t-test	Age	54	4.176	0.0001	***	28	N/A
3a	Comparison	Young vs aged wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Age	1, 54	13.13	0.0006	***	28	Young vs Aged: 3-4, 10, 43-53 and 62-67Hz
						Frequency	79, 4266	8.031	<0.0001	****		
						Age x Frequency	79, 4266	8.031	<0.0001	****		
3b	Comparison	Young vs aged NREM qEEG	% change from young	Active	Repeated Measures Two-Way ANOVA	Age	1, 54	2.339	0.1320	ns	28	Young vs Aged: 73-79Hz
						Frequency	79, 4266	6.552	<0.0001	****		
						Age x Frequency	79, 4266	6.552	<0.0001	****		
3c	Comparison	Young vs aged gamma power during wake	% change from young	Active	Repeated Measures Two-Way ANOVA	Age	1, 54	23.07	<0.0001	****	28	Young vs Aged: -2, 0, 1, 2, 3, 4, 5, 6, 7 and 8Hr
						Time	10, 540	2.461	0.0070	**		
						Age x Time	10, 540	2.461	0.0070	**		
3d	Comparison	Young vs aged NREM delta (SWA)	% change from young	Active	Repeated Measures Mixed-Effects Model (REML)	Age	1, 54	0.2870	0.5943	ns	28	Young vs Aged: -2Hr
						Time	10, 522	0.6589	<0.0001	****		
						Age x Time	10, 522	4.314	<0.0001	****		
3e	Comparison	Young vs aged wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 52	3.955	0.1383	ns	27	Young vs Aged: 3 and 9Hz
						Frequency	79, 4108	5.940	<0.0001	****		
						Age x Frequency	79, 4108	5.940	<0.0001	****		
3f	Comparison	Young vs aged NREM qEEG	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 52	1.294	0.2606	ns	27	None
						Frequency	79, 4108	3.024	<0.0001	****		
						Age x Frequency	79, 4108	3.024	<0.0001	****		
3g	Comparison	Young vs aged REM qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Age	1,50	0.1427	0.7072	ns	26	Young vs Aged: 34, 54, 56-59, 76-79Hz
						Frequency	79, 3950	6.546	<0.0001	****		
						Age x Frequency	79, 3950	6.546	<0.0001	****		
3h	Comparison	Young vs aged gamma power during wake	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 52	0.6150	0.4365	ns	27	N/A
						Time	10, 520	0.7216	0.7044	ns		
						Age x Time	10, 520	0.7216	0.7044	ns		
3i	Comparison	Young vs aged NREM delta (SWA)	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 52	0.001702	0.9673	ns	27	None
						Time	10, 520	5.666	<0.0001	****		
						Age x Time	10, 520	5.666	<0.0001	****		

4a	Young	Xanomeline effects on time in w ake	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	12.83	<0.0001	****	14	10 mg/kg vs Veh time: ZT18 30 mg/kg vs Veh time: ZT14, 20 and 0
						Time	11, 143	88.02	<0.0001	****		
						Dose x Time	33, 429	4.034	<0.0001	****		
4b	Young	Xanomeline effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	17.07	<0.0001	****	14	10 mg/kg vs Veh time: ZT18 30 mg/kg vs Veh time: ZT14, 20 and 0
						Time	11, 143	83.82	<0.0001	****		
						Dose x Time	33, 429	4.052	<0.0001	****		
4c	Young	Xanomeline effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	2.002	0.1296	ns	14	3 mg/kg vs Veh time: ZT0 30 mg/kg vs Veh time: ZT20
						Time	11, 143	80.78	<0.0001	****		
						Dose x Time	33, 429	2.95	<0.0001	****		
4d	Young	Xanomeline effects on time in w ake	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	12.83	<0.0001	****	14	3 mg/kg vs Veh time: ZT12-24 10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT0-12
						Time	1, 13	866.8	<0.0001	****		
						Dose x Time	3, 39	8.373	0.0002	***		
4e	Young	Xanomeline effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	17.07	<0.0001	****	14	3 mg/kg vs Veh time: ZT12-24 10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24 and 0-12
						Time	1, 13	971.8	<0.0001	****		
						Dose x Time	3, 39	6.445	0.0012	**		
4f	Young	Xanomeline effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	2.002	0.1296	ns	14	3 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT12-24
						Time	1, 13	334.6	<0.0001	****		
						Dose x Time	3, 39	10.18	<0.0001	****		
4g	Aged	Xanomeline effects on time in Wake	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	28	<0.0001	****	14	3 mg/kg vs Veh time: ZT12 and 14 10 mg/kg vs Veh time: ZT10 30 mg/kg vs Veh time: ZT14, 16 and 20
						Time	11, 143	100.2	<0.0001	****		
						Dose x Time	33, 429	4.965	<0.0001	****		
4h	Aged	Xanomeline effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	15.09	<0.0001	****	14	3 mg/kg vs Veh time: ZT14, 20 , 0 and 2 10 mg/kg vs Veh time: ZT0, 2, 6, and 10 30 mg/kg vs Veh time: ZT14 amd 16
						Time	11, 143	106.3	<0.0001	****		
						Dose x Time	33, 429	6.313	<0.0001	****		
4i	Aged	Xanomeline effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	1.121	0.3523	ns	14	10 mg/kg vs Veh time: ZT4 30 mg/kg vs Veh time: ZT2
						Time	11, 143	157	<0.0001	****		
						Dose x Time	33, 429	1.848	0.0035	**		
4j	Aged	Xanomeline effects on time in w ake	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	28.00	<0.0001	****	14	3 mg/kg vs Veh time: ZT12-24, 0-12 10 mg/kg vs Veh time: ZT12-24
						Time	1, 13	385.3	<0.0001	****		
						Dose x Time	3, 39	0.5362	0.6602	ns		
4k	Aged	Xanomeline effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	15.09	<0.0001	****	14	3 mg/kg vs Veh time: ZT0-12 10 mg/kg vs Veh time: ZT0-12
						Time	1, 13	475.8	<0.0001	****		
						Dose x Time	3, 39	1.737	0.1754	ns		
4l	Aged	Xanomeline effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	1.121	0.3523	ns	14	3 mg/kg vs Veh time: ZT0-12 10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12
						Time	1, 13	630.1	<0.0001	****		
						Dose x Time	3, 39	5.411	0.0033	**		
5a	Young	Xanomeline effects on w ake bout number	Bout number (ZT14 - ZT22)	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	0.5402	0.6576	ns	14	N/A
5b	Young	Xanomeline effects on w ake bout duration	Average bout duration (ZT14 -	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	2.159	0.1084	ns	14	N/A
5c	Aged	Xanomeline effects on w ake bout number	Bout number (ZT14 - ZT22)	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	14.45	<0.0001	****	14	3 mg/kg vs Veh bout number: p=0.0003 10 mg/kg vs Veh bout number: p=0.0090 30 mg/kg vs Veh bout number: p<0.0001
5d	Aged	Xanomeline effects on w ake bout duration	Average bout duration (ZT14 -	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	11.21	<0.0001	****	14	30 mg/kg vs Veh bout number: p<0.0001
6a	Young	Xanomeline effects on NREM sleep bout number	Bout number (ZT14 - ZT22)	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	1.255	0.3031	ns	14	N/A
6b	Young	Xanomeline effects on NREM sleep bout duration	Average bout duration (ZT14 -	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	1.017	0.3956	ns	14	N/A
6c	Aged	Xanomeline effects on NREM sleep bout number	Bout number (ZT14 - ZT22)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	11.88	<0.0001	****	14	3 mg/kg vs Veh bout number: p=0.0008 10 mg/kg vs Veh bout number: p=0.0209 30 mg/kg vs Veh bout number: p<0.0001
6d	Aged	Xanomeline effects on NREM sleep bout duration	Average bout duration (ZT14 -	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	11.94	<0.0001	****	14	3 mg/kg vs Veh bout number: p<0.0001 10 mg/kg vs Veh bout number: p=0.0040 30 mg/kg vs Veh bout number: p<0.0001

7a	Young	Donepezil effects on time in w ake	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	1.216	0.3154	ns	14	0.1mg/kg vs Veh time: ZT18 3 mg/kg vs Veh time: ZT14 and 16
						Time	11, 143	118.7	<0.0001	****		
						Dose x Time	44, 572	1.964	0.0003	***		
7b	Young	Donepezil effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	0.9213	0.4587	ns	14	0.1mg/kg vs Veh time: ZT18 1mg/kg vs Veh time: ZT12 3 mg/kg vs Veh time: ZT14 and 16
						Time	11, 143	110.3	<0.0001	****		
						Dose x Time	44, 572	0.9213	0.4587	ns		
7c	Young	Donepezil effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	1.316	0.2762	ns	14	0.1mg/kg vs Veh time: ZT18 0.3 mg/kg vs Veh time: ZT18
						Time	11, 143	131.9	<0.0001	****		
						Dose x Time	44, 572	1.506	0.0216	*		
7d	Young	Donepezil effects on time in w ake	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	1.216	0.3154	ns	14	N/A
						Time	1, 13	1308	<0.0001	****		
						Dose x Time	4, 52	1.322	0.2740	ns		
7e	Young	Donepezil effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4,52	0.9213	0.4587	ns	14	N/A
						Time	1, 13	1294	<0.0001	****		
						Dose x Time	4, 52	1.623	0.1824	ns		
7f	Young	Donepezil effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	1.316	0.2762	ns	14	N/A
						Time	1, 13	784.3	<0.0001	****		
						Dose x Time	4, 52	0.9867	0.4230	ns		
7g	Aged	Donepezil effects on time in Wake	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	0.7397	0.5692	ns	14	1mg/kg vs Veh time: ZT12
						Time	11, 143	97.07	<0.0001	****		
						Dose x Time	44, 572	2.009	0.0002	***		
7h	Aged	Donepezil effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	0.6487	0.6304	ns	14	None
						Time	11, 143	95.84	<0.0001	****		
						Dose x Time	44, 572	1.867	0.0008	***		
7i	Aged	Donepezil effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	4.509	0.0033	**	14	1mg/kg vs Veh time: ZT16 and 18 3 mg/kg vs Veh time: ZT 20 and 4
						Time	11, 143	107.5	<0.0001	****		
						Dose x Time	44, 572	1.815	0.0014	**		
7j	Aged	Donepezil effects on time in w ake	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	0.7397	0.5692	ns	14	N/A
						Time	1, 13	358.8	<0.0001	****		
						Dose x Time	4, 52	1.708	0.1623	ns		
7k	Aged	Donepezil effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	0.6487	0.6304	ns	14	N/A
						Time	1, 13	343.7	<0.0001	****		
						Dose x Time	4, 52	1.522	0.2095	ns		
7l	Aged	Donepezil effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	4.509	0.0033	**	14	None
						Time	1, 13	350.2	<0.0001	****		
						Dose x Time	4, 52	1.648	0.1763	ns		
8a	Young	Xanomeline effects on time in w ake	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	7.111	0.0006	***	14	3 mg/kg vs Veh time: ZT4 10 mg/kg vs Veh time: ZT2, 4 and 18 30 mg/kg vs Veh time: ZT2, 4, 14, 16 and 18
						Time	11, 143	104.6	<0.0001	****		
						Dose x Time	33, 429	10.79	<0.0001	****		
8b	Young	Xanomeline effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	7.145	0.0006	***	14	3 mg/kg vs Veh time: ZT2 and 4 10 mg/kg vs Veh time: ZT2, 4 and 18 30 mg/kg vs Veh time: ZT2, 4, 14, 16 and 18
						Time	11, 143	96.51	<0.0001	****		
						Dose x Time	33, 429	10.36	<0.0001	****		
8c	Young	Xanomeline effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	0.4242	0.7367	ns	14	10 mg/kg vs Veh time: ZT2 30 mg/kg vs Veh time: ZT2, 4, 8, 14, 18 and 20
						Time	11, 143	86.9	<0.0001	****		
						Dose x Time	33, 429	10.4	<0.0001	****		
8d	Young	Xanomeline effects on time in w ake	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	7.111	0.0006	***	14	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12, 12-24
						Time	1, 13	894.6	<0.0001	****		
						Dose x Time	3, 39	69.09	<0.0001	****		
8e	Young	Xanomeline effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	7.145	0.0006	***	14	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12, 12-24
						Time	1, 13	950.2	<0.0001	****		
						Dose x Time	3, 39	65.23	<0.0001	****		
8f	Young	Xanomeline effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	0.7367	0.7367	ns	14	30 mg/kg vs Veh time: ZT0-12, 12-24
						Time	1, 13	<0.0001	<0.0001	****		
						Dose x Time	3, 39	<0.0001	<0.0001	****		
8g	Aged	Xanomeline effects on time in Wake	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	5.796	0.0024	**	13	10 mg/kg vs Veh time: ZT2, 12, 14, 18 and 20 30 mg/kg vs Veh time:ZT2, 4, 6, 12, 14, 16, 18 and 20
						Time	100.2	56.92	<0.0001	****		
						Dose x Time	33, 429	21.28	<0.0001	****		
8h	Aged	Xanomeline effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 36	6.676	0.0011	**	13	10 mg/kg vs Veh time: ZT2, 12, 14 and 20 30 mg/kg vs Veh time: ZT2, 4, 12, 14, 16, 18 and 20
						Time	11, 132	51.6	<0.0001	****		
						Dose x Time	33, 396	19.6	<0.0001	****		

8i	Aged	Xanomeline effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	0.7242	0.5442	ns	13	10 mg/kg vs Veh time: ZT2, 4, 14, 16, 18 and 20 30 mg/kg vs Veh time: ZT2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22
						Time	11, 132	47.35	<0.0001	****		
						Dose x Time	33, 396	17.3	<0.0001	****		
8j	Aged	Xanomeline effects on time in wake	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	5.796	<0.0001	****	13	10 mg/kg vs Veh time: ZT0-12, 12-24 30 mg/kg vs Veh time: ZT0-12, 12-24
						Time	1, 12	205.5	0.0024	**		
						Dose x Time	3, 36	78.31	<0.0001	****		
8k	Aged	Xanomeline effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	6.676	0.0011	**	13	3 mg/kg vs Veh time: ZT12-24 10 mg/kg vs Veh time: ZT0-12, 12-24 30 mg/kg vs Veh time: ZT0-12, 12-24
						Time	1, 12	195.6	<0.0001	****		
						Dose x Time	3, 36	72.32	<0.0001	****		
8l	Aged	Xanomeline effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	0.7242	0.5442	ns	13	10 mg/kg vs Veh time: ZT0-12, 12-24 30 mg/kg vs Veh time: ZT0-12, 12-24
						Time	1, 12	106.6	<0.0001	****		
						Dose x Time	3, 36	54.90	<0.0001	****		
9a	Young	Donepezil effects on time in wake	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	1.654	0.1761	ns	13	3 mg/kg vs Veh time: ZT2, 12, 14 and 20
						Time	11, 132	83.34	<0.0001	****		
						Dose x Time	44, 528	3.48	<0.0001	****		
9b	Young	Donepezil effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	1.555	0.2016	ns	13	0.1mg/kg vs Veh time: ZT8 3 mg/kg vs Veh time: ZT2, 4, 8, 12 and 20
						Time	11, 132	70.58	<0.0001	****		
						Dose x Time	44, 528	3.319	<0.0001	****		
9c	Young	Donepezil effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	0.4723	0.7558	ns	13	0.1mg/kg vs Veh time: ZT4 and 10 1mg/kg vs Veh time: ZT4 3 mg/kg vs Veh time: ZT2 and 20
						Time	11, 132	100.4	<0.0001	****		
						Dose x Time	44, 528	2.39	<0.0001	****		
9d	Young	Donepezil effects on time in wake	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	1.654	0.1761	ns	13	0.1mg/kg vs Veh time: ZT12-24 3 mg/kg vs Veh time: ZT0-12 and 12-24
						Time	1, 12	202.1	<0.0001	****		
						Dose x Time	4, 48	12.33	<0.0001	****		
9e	Young	Donepezil effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	1.555	0.2016	ns	13	0.1mg/kg vs Veh time: ZT12-24 3 mg/kg vs Veh time: ZT0-12 and 12-24
						Time	1, 12	183.1	<0.0001	****		
						Dose x Time	4, 48	11.22	<0.0001	****		
9f	Young	Donepezil effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	0.4723	0.7558	ns	13	3 mg/kg vs Veh time: ZT12-24
						Time	1, 12	233.5	<0.0001	****		
						Dose x Time	4, 48	2.696	0.0417	*		
9g	Aged	Donepezil effects on time in Wake	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.652	0.0433	*	14	3 mg vs Veh time: ZT2, 4 and 14
						Time	11 ,143	75.92	<0.0001	****		
						Dose x Time	44, 572	5.445	<0.0001	****		
9h	Aged	Donepezil effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	3.782	0.0090	**	14	3 mg vs Veh time: ZT2, 4 and 22
						Time	11 ,143	73.02	<0.0001	****		
						Dose x Time	44, 572	5.016	<0.0001	****		
9i	Aged	Donepezil effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	3.156	0.0214	*	14	1mg/kg vs Veh time: ZT 0 3 mg vs Veh time: ZT0, 2, 4, 10, 14, 16 and 18
						Time	11 ,143	51.14	<0.0001	****		
						Dose x Time	44, 572	4.204	<0.0001	****		
9j	Aged	Donepezil effects on time in wake	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.652	0.0433	*	14	3 mg vs Veh time: ZT0-12 and 12-24
						Time	1 ,13	236.1	<0.0001	****		
						Dose x Time	4, 52	13.99	<0.0001	****		
9k	Aged	Donepezil effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	3.782	0.0090	**	14	3 mg vs Veh time: ZT0-12
						Time	1 ,13	214.4	<0.0001	****		
						Dose x Time	4, 52	14.07	<0.0001	****		
9l	Aged	Donepezil effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	3.156	0.0214	*	14	3 mg vs Veh time: ZT12-24
						Time	1 ,13	201.1	<0.0001	****		
						Dose x Time	4, 52	6.063	0.0004	***		
10a	Young	Xanomeline effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	6.375	0.0725	ns	14	10 mg/kg vs Veh Freq: 9-11, 29-31, 33, 36, 53 and 56-60Hz 30 mg/kg vs Veh Freq: 0.5-1, 3-6, 8-13, 23, 38-45, 51-58 and 64-79Hz
						Frequency	79, 1027	2.515	<0.0001	****		
						Dose x Frequency	237, 3081	7.602	<0.0001	****		
10b	Young	Xanomeline effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	12.27	<0.0001	****	13-14	10 mg/kg vs Veh Freq: 0.5-1Hz 30 mg/kg vs Veh Freq: 0.5-2, 4-5 and 31-79Hz
						Frequency	79, 1027	13.22	<0.0001	****		
						Dose x Frequency	237, 2921	13.07	<0.0001	****		
10c	Young	Xanomeline effects on wake gamma power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.702	0.1824	ns	14	10 mg/kg vs Veh Gamma Power: Time relative to dose: 0Hr 30 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 1, 2Hr
						Time	10 ,130	8.332	<0.0001	****		
						Dose x Time	30, 390	2.022	0.0014	**		
10d	Young	Xanomeline effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.9823	0.4110	ns	14	10 mg/kg vs Veh SWA: Time relative to dose: 0, 4Hr 30 mg/kg vs Veh SWA: Time relative to dose: 0, 6, 8Hr
						Time	10 ,130	41.67	<0.0001	****		
						Dose x Time	30, 329	4.101	<0.0001	****		

10e	Aged	Xanomeline effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	7.664	0.0004	***	14	3 mg/kg vs Veh Freq: 2-3Hz 10 mg/kg vs Veh Freq: 2, 56-65 and 68-69Hz 30 mg/kg vs Veh Freq: 0.5-1, 2-4, 6-12 and 18-53Hz
						Frequency	79, 1027	8.046	<0.0001	****		
						Dose x Frequency	237, 3081	12.14	<0.0001	****		
10f	Aged	Xanomeline effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	0.1943	0.8246	ns	12-14	N/A
						Frequency	79, 1027	4.038	<0.0001	****		
						Dose x Frequency	158, 1894	1.012	0.4447	ns		
10g	Aged	Xanomeline effects on wake gamma power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.187	0.0116	*	14	3 mg/kg vs Veh Gamma Power: Time relative to dose: 1, 2, 3, 4, 5, 6, 7, 8Hr 10 mg/kg vs Veh Gamma Power: Time relative to dose: 1, 2, 3, 4, 5, 6, 7, 8Hr 30 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 1, 2, 3, 4, 5, 6, 7Hr
						Time	10, 130	7.946	<0.0001	****		
						Dose x Time	30, 390	5.963	<0.0001	****		
10h	Aged	Xanomeline effects on NREM delta (SWA) (-2 to 2hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	3.980	0.0311	*	8-14	10 mg/kg vs Veh SWA: Time relative to dose: 0, 1Hr
						Time	4, 52	2.627	0.0448	*		
						Dose x Time	8, 91	8.341	<0.0001	****		
	Aged	Xanomeline effects on NREM delta (SWA) (3 to 8hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.9067	0.4466	ns	12-14	N/A
						Time	5, 65	2.41	0.0457	*		
						Dose x Time	15, 185	0.7921	0.6855	ns		
11a	Young	Xanomeline effects on wake delta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	10.88	<0.0001	****	14	30 mg/kg vs veh delta: 0, 5, 8Hr
						Time	10, 130	24.00	<0.0001	****		
						Dose x Time	30, 390	17.27	<0.0001	****		
11b	Aged	Xanomeline effects on wake delta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.962	0.0147	*	14	3 mg/kg vs veh delta: 0, 1, 2, 3, 4, 5, 6, 7 and 8Hr 10 mg/kg vs veh delta: 1, 2, 3, 5 and 7Hr 30 mg/kg vs veh delta: 0, 1, 2, 3, 4, 5, 6 and 7Hr
						Time	10, 130	10.43	<0.0001	****		
						Dose x Time	3, 390	14.16	<0.0001	****		
11c	Young	Xanomeline effects on wake theta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.430	0.0090	**	14	10 mg/kg vs veh theta: 0, 1and 4Hr 30 mg/kg vs veh theta: 1, 2, 3, 6 and 8Hr
						Time	10, 130	5.900	<0.0001	****		
						Dose x Time	30, 390	9.976	<0.0001	****		
11d	Aged	Xanomeline effects on wake theta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	0.4906	0.6909	ns	14	10 mg/kg vs veh theta: 0Hr 30 mg/kg vs veh theta: 0, 1, 3, 4 and 5Hr
						Time	10, 130	12.36	<0.0001	****		
						Dose x Time	30, 390	26.51	<0.0001	****		
11e	Young	Xanomeline effects on wake alpha power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	11.40	<0.0001	****	14	3 mg/kg vs veh alpha: 0Hr 10 mg/kg vs veh alpha: 0, 1, 5 and 8Hr 30 mg/kg vs veh alpha: 0, 1, 2, 5, 6 and 8Hr
						Time	10, 130	25.26	<0.0001	****		
						Dose x Time	30, 390	15.08	<0.0001	****		
11f	Aged	Xanomeline effects on wake alpha power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	10.57	<0.0001	****	14	10 mg/kg vs veh alpha: 0 and 1Hr 30 mg/kg vs veh alpha: 0, 1, 2 and 8Hr
						Time	10, 130	27.24	<0.0001	****		
						Dose x Time	30, 390	23.90	<0.0001	****		
11g	Young	Xanomeline effects on wake beta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	2.754	0.0553	ns	14	10 mg/kg vs veh beta: 5 and 6Hr 30 mg/kg vs veh beta: 0, 2, 3, 4, 5 and 8Hr
						Time	10, 130	3.752	0.0002	***		
						Dose x Time	30, 390	8.451	<0.0001	****		
11h	Aged	Xanomeline effects on wake beta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.299	0.0103	*	14	10 mg/kg vs veh beta: 0Hr 30 mg/kg vs veh beta: 0, 1, 2, and 7Hr
						Time	10, 130	9.618	<0.0001	****		
						Dose x Time	30, 390	7.588	<0.0001	****		
12a	Young	Xanomeline effects on NREM theta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.547	0.2176	ns	5-14	10 mg/kg vs veh theta: 0 and 1Hr 30 mg/kg vs veh theta: 0, 1and 2Hr
						Time	10, 130	19.22	<0.0001	****		
						Dose x Time	30, 329	10.26	<0.0001	****		
12b	Aged	Xanomeline effects on NREM theta power (-2 to 2hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	12.21	0.0002	***	8-14	10 mg/kg vs veh theta: 0 and 1Hr
						Time	4, 52	0.8815	0.4815	ns		
						Dose x Time	8, 91	15.99	<0.0001	****		
	Aged	Xanomeline effects on NREM theta power (3 to 8hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	5.464	0.0031	**	13-14	30 mg/kg vs veh theta: 3, 4, 5 and 6Hr
						Time	5, 65	0.8877	0.4946	ns		
						Dose x Time	15, 185	2.163	0.0090	**		
12c	Young	Xanomeline effects on NREM alpha power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.4929	0.6893	ns	5-14	10 mg/kg vs veh alpha: 0, 4Hr 30 mg/kg vs veh alpha: 0, 1, 6 and 7Hr
						Time	10, 130	33.77	<0.0001	****		
						Dose x Time	30, 329	11.70	<0.0001	****		
12d	Aged	Xanomeline effects on NREM alpha power (-2 to 2hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	0.4918	0.6171	ns	8-14	10 mg/kg vs veh alpha: 0Hr
						Time	4, 52	69.78	<0.0001	****		
						Dose x Time	8, 91	3.351	0.0021	**		
	Aged	Xanomeline effects on NREM alpha power (3 to 8hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	11.73	0.1071	ns	13-14	30 mg/kg vs veh alpha: 3, 5, 6, 7 and 8Hr
						Time	5, 65	2.261	<0.0001	****		
						Dose x Time	15, 185	7.013	<0.0001	****		

12e	Young	Xanomeline effects on NREM beta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.08236	0.9692	ns	5-14	10 mg/kg vs veh beta: 0Hr 30 mg/kg vs veh beta: 0Hr
						Time	10, 130	28.39	<0.0001	****		
						Dose x Time	30, 329	2.488	<0.0001	****		
12f	Aged	Xanomeline effects on NREM beta power (-2 to 2hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	0.4943	0.6156	****	8-14	10 mg/kg vs veh beta: 0Hr
						Time	4, 52	8.379	<0.0001	ns		
						Dose x Time	8, 91	2.428	0.0200	*		
	Aged	Xanomeline effects on NREM beta power (3 to 8hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.613	0.1108	ns	13-14	N/A
						Time	5, 65	1.875	0.2019	ns		
						Dose x Time	15, 185	1.187	0.2848	ns		
12g	Young	Xanomeline effects on NREM gamma power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	15.40	<0.0001	****	5-14	10 mg/kg vs veh gamma: 0Hr 30 mg/kg vs veh gamma: 0, 1and 2Hr
						Time	10, 130	25.73	<0.0001	****		
						Dose x Time	30, 329	13.55	<0.0001	****		
12h	Aged	Xanomeline effects on NREM gamma power (-2 to 2hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	0.9918	0.3845	ns	8-14	10 mg/kg vs veh beta: 0Hr
						Time	4, 52	1.218	0.3145	ns		
						Dose x Time	8, 91	7.747	<0.0001	****		
	Aged	Xanomeline effects on NREM gamma power (3 to 8hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	2.401	,0.0824	ns	13-14	N/A
						Time	5, 65	1.571	0.1807	ns		
						Dose x Time	15, 185	1.683	0.0573	ns		
13a	Young	Donepezil effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.574	<0.0001	****	14	3 mg/kg vs Veh Freq: 2-3, 7, 9-12, 20-38Hz
						Frequency	79, 1027	3.755	0.0483	*		
						Dose x Frequency	316, 4108	2.478	<0.0001	****		
13b	Young	Donepezil effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	0.4812	0.7494	ns	12-14	3 mg/kg vs Veh Freq: 3Hz
						Frequency	79, 1027	32.39	<0.0001	****		
						Dose x Frequency	316, 3788	1.168	0.0260	*		
13c	Young	Donepezil effects on wake gamma power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.267	0.0744	ns	14	0.1mg/kg vs Veh Gamma Power: Time relative to dose: 4Hr 1mg/kg vs Veh Gamma Power: Time relative to dose: 0Hr 3 mg/kg vs Veh Gamma Power: Time relative to dose: 0Hr
						Time	10, 130	15.23	<0.0001	****		
						Dose x Time	4, 520	1.756	0.0035	**		
13d	Young	Donepezil effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	0.8698	0.4884	ns	14	None
						Time	40, 474	32.44	<0.0001	****		
						Dose x Time	10, 130	1.762	0.0035	**		
13e	Aged	Donepezil effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	0.9345	0.4513	ns	14	0.1mg/kg vs Veh Freq: 4Hz 1mg/kg vs Veh Freq: 8-9Hz 3 mg/kg vs Veh Freq: 2-4, 7-11, 19-28 and 73-79Hz
						Frequency	79, 1027	5.099	<0.0001	****		
						Dose x Frequency	316, 4108	3.389	<0.0001	****		
13f	Aged	Donepezil effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	5.221	0.0013	**	13-14	3 mg/kg vs Veh Freq: 0.5-1, 41-43, 47-53, 56-57 and 61-79Hz
						Frequency	79, 1027	4.675	<0.0001	****		
						Dose x Frequency	316, 3948	3.822	<0.0001	****		
13g	Aged	Donepezil effects on wake gamma power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	0.1591	0.9580	ns	14	N/A
						Time	10, 130	10.54	<0.0001	****		
						Dose x Time	40, 520	0.8782	0.6851	ns		
13h	Aged	Donepezil effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	10, 130	2.875	0.0316	*	14	1mg/kg vs Veh SWA: Time relative to dose: 5, 8Hr
						Time	4, 52	8.789	<0.0001	****		
						Dose x Time	40, 503	2.144	<0.0001	****		
14a	Young	Donepezil effects on wake delta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	5.329	<0.0001	****	14	1mg/kg vs veh delta: 5Hr 3 mg/kg vs veh delta: 0 and 1Hr
						Time	10, 130	12.47	0.0011	**		
						Dose x Time	40, 520	4.536	<0.0001	****		
14b	Aged	Donepezil effects on wake delta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	4.274	0.0046	**	14	3 mg/kg vs veh delta: 0 and 1Hr
						Time	10, 130	10.05	<0.0001	****		
						Dose x Time	40, 520	4.966	<0.0001	****		
14c	Young	Donepezil effects on wake theta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.491	0.0543	ns	14	N/A
						Time	10, 130	14.39	<0.0001	****		
						Dose x Time	40, 520	1.378	0.0653	ns		
14d	Aged	Donepezil effects on wake theta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.521	0.0521	ns	14	1mg/kg vs veh theta: 5 and 6Hr 3 mg/kg vs veh theta: 0Hr
						Time	10, 130	3.458	0.0005	***		
						Dose x Time	40, 520	1.579	0.0152	*		
14e	Young	Donepezil effects on wake alpha power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	8.133	<0.0001	****	14	0.1mg/kg vs veh alpha: 4Hr 1mg/kg vs veh alpha: 0, 1and 2Hr 3 mg/kg vs veh alpha: 0, 1, 2 and 4Hr
						Time	10, 130	8.327	<0.0001	****		
						Dose x Time	40, 520	9.084	<0.0001	****		
14f	Aged	Donepezil effects on wake alpha power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.065	0.0988	ns	14	1mg/kg vs veh alpha: 0 and 4Hr 3 mg/kg vs veh alpha: 0 and 1Hr
						Time	10, 130	6.460	<0.0001	****		
						Dose x Time	40, 520	4.931	<0.0001	****		

14g	Young	Donepezil effects on wake beta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	6.006	0.0005	***	14	0.1mg/kg vs veh beta: 4Hr 1mg/kg vs veh beta: 0 and 2Hr 3 mg/kg vs veh beta: 0, 1and 2Hr
						Time	10, 130	15.88	<0.0001	****		
						Dose x Time	40, 520	6.651	<0.0001	****		
14h	Aged	Donepezil effects on wake beta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.272	0.0739	ns	14	1mg/kg vs veh beta:0Hr 3 mg/kg vs veh beta: 0 and 1Hr
						Time	10, 130	16.68	<0.0001	****		
						Dose x Time	40, 520	6.697	<0.0001	****		
15a	Young	Donepezil effects on NREM theta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	0.3170	0.8654	ns	10-14	N/A
						Time	10, 130	26.96	<0.0001	****		
						Dose x Time	40, 474	1.320	0.0960	ns		
15b	Aged	Donepezil effects on NREM theta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	2.222	0.0792	ns	11-14	0.1mg/kg vs veh theta: 0Hr 1mg/kg vs veh theta: 8Hr
						Time	10, 130	4.974	<0.0001	****		
						Dose x Time	40, 503	1.749	0.0038	**		
15c	Young	Donepezil effects on NREM alpha power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	1.643	0.1775	ns	10-14	0.3 mg/kg vs veh alpha: -2Hr 3 mg/kg vs veh alpha: 1Hr
						Time	10, 130	25.19	<0.0001	****		
						Dose x Time	40, 474	1.929	0.0008	***		
15e	Aged	Donepezil effects on NREM alpha power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	4.039	0.0063	**	11-14	0.1mg/kg vs veh beta: 2, 5, 6 and 8Hr 0.3 mg/kg vs veh beta: 5 and 7Hr 1mg/kg vs veh beta: 2, 4, 5, 6 and 7Hr 3 mg/kg vs veh beta: 5, 6, 7 and 8Hr
						Time	10, 130	16.22	<0.0001	****		
						Dose x Time	40, 503	2.298	<0.0001	****		
15f	Young	Donepezil effects on NREM beta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4.52	1.563	0.1979	ns	10-14	0.1mg/kg vs veh beta: 0Hr 0.3 mg/kg vs veh beta: -2 and 0Hr
						Time	10, 130	33.88	<0.0001	****		
						Dose x Time	40, 474	1.874	0.0013	**		
15g	Aged	Donepezil effects on NREM beta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	3.035	0.0253	*	11-14	1mg/kg vs veh alpha: 8Hr
						Time	10, 130	9.971	<0.0001	****		
						Dose x Time	40, 503	2.181	<0.0001	****		
15h	Young	Donepezil effects on NREM gamma power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	1.391	0.2498	ns	10-14	0.1mg/kg vs veh gamma: 0Hr 0.3 mg/kg vs veh gamma: -2Hr 3 mg/kg vs veh gamma: 0 and 4Hr
						Time	10, 130	17.71	<0.0001	****		
						Dose x Time	40, 474	3.721	<0.0001	****		
15i	Aged	Donepezil effects on NREM gamma power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	5.337	0.0011	**	11-14	0.1mg/kg vs veh gamma: 0Hr 1mg/kg vs veh gamma: 8Hr 3 mg/kg vs veh gamma: 0 and 1Hr
						Time	10, 130	4.034	<0.0001	****		
						Dose x Time	40, 503	4.845	<0.0001	****		
16a	Young	Xanomeline effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.63	0.0211	*	14	3 mg/kg vs Veh Freq: 1-3, 9-11, 40-44, 46, 64-65, 67-74 and 78-79Hz 10 mg/kg vs Veh Freq: 2-4, 39-49, 51-52 and 54-79Hz 30 mg/kg vs Veh Freq: 0.5-2, 4-5 and 33-79Hz
						Frequency	79, 1027	6.208	<0.0001	****		
						Dose x Frequency	237, 3081	4.912	<0.0001	****		
16b	Young	Xanomeline effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3,39	17.17	<0.0001	****	13-14	3 mg/kg vs Veh Freq: 0.5-1Hz 10 mg/kg vs Veh Freq: 0.5-1Hz 30 mg/kg vs Veh Freq: 0.5-1, 3-6, 9-14 and 28-79Hz
						Frequency	79, 1027	50.28	<0.0001	****		
						Dose x Frequency	237, 3001	26.72	<0.0001	****		
16c	Young	Xanomeline effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	0.2138	0.8089	ns	13-14	3 mg/kg vs Veh Freq: 5 and 11Hz 10 mg/kg vs Veh Freq: 0.5-5 and 7Hz 30 mg/kg: insufficient mice displayed REM sleep
						Frequency	79, 1027	3.219	<0.0001	****		
						Dose x Frequency	158, 1894	1.826	<0.0001	****		
16d	Young	Xanomeline effects on wake gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.415	0.0266	*	14	3 mg/kg vs Veh Gamma Power: Time relative to dose: -1, 0Hr 10 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 1Hr 30 mg/kg vs Veh Gamma Power: Time relative to dose: 1, 2, 3Hr
						Time	10, 130	10.16	<0.0001	****		
						Dose x Time	30, 390	2.998	<0.0001	****		
16e	Young	Xanomeline effects on NREM delta (SWA) (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	26.81	0.0163	*	13-14	3 mg/kg vs Veh SWA: Time relative to dose: 0Hr 10 mg/kg vs Veh SWA: Time relative to dose: 0Hr
						Time	2, 26	4.847	<0.0001	****		
						Dose x Time	4, 51	17.76	<0.0001	****		
	Young	Xanomeline effects on NREM delta (SWA) (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	3.327	0.0293	*	13-14	3 mg/kg vs Veh SWA: Time relative to dose: 1Hr 10 mg/kg vs Veh SWA: Time relative to dose: 1Hr 30 mg/kg vs Veh SWA: Time relative to dose: 1Hr
						Time	7, 91	28.78	<0.0001	****		
						Dose x Time	21, 272	1.494	<0.0001	****		
16f	Aged	Xanomeline effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	22.46	<0.0001	****	13	10 mg/kg vs Veh Freq: 3 and 43Hz 30 mg/kg vs Veh Freq: 0.5-1, 2, 6-12, 18-59 and 61-63Hz
						Frequency	79, 948	13.16	<0.0001	****		
						Dose x Frequency	237, 2844	13.68	<0.0001	****		
16g	Aged	Xanomeline effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	2, 24	1.650	0.2131	ns	13	3 mg/kg vs Veh Freq: 0.5-1, 55 and 79Hz 10 mg/kg vs Veh Freq: 0.5-1, 3-5, 8-14, 23-26, 38, 40-42, 50-54 and 56-79Hz 30 mg/kg: insufficient mice displayed NREM sleep
						Frequency	79, 948	2.760	<0.0001	****		
						Dose x Frequency	158, 1896	7.059	<0.0001	****		
16h	Aged	Xanomeline effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 24	0.0001312	0.9999	ns	7-12	10 mg/kg vs Veh Freq: 2-3, 6, 8-12, 28 and 30Hz 30 mg/kg: insufficient mice displayed REM sleep
						Frequency	79, 948	2.768	<0.0001	****		
						Dose x Frequency	158, 1256	1.498	0.0020	***		
16i	Aged	Xanomeline effects on wake gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	5.081	0.0049	**	13	3 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 3Hr 10 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 1Hr 30 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 1, 2, 3Hr
						Time	10, 120	18.87	<0.0001	****		
						Dose x Time	30, 360	11.25	<0.0001	****		
16j	Aged	Xanomeline effects on NREM delta (SWA) (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 24	17.32	<0.0001	****	9-13	3 mg/kg vs Veh SWA: Time relative to dose: 0Hr 10 mg/kg vs Veh SWA: Time relative to dose: 0, 1Hr
						Time	3, 36	13.62	<0.0001	****		
						Dose x Time	6, 68	15.83	<0.0001	****		
	Aged	Xanomeline effects on NREM delta (SWA) (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 36	4.600	0.0080	**	7-13	30 mg/kg vs Veh SWA: Time relative to dose: 2, 3, 4, 5Hr
						Time	6, 72	9.321	<0.0001	****		
						Dose x Time	18, 210	3.999	<0.0001	****		

17a	Young	Xanomeline effects on wake delta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.455	0.2416	ns	14	3 mg/kg vs veh delta: 1Hr 10 mg/kg vs veh delta: 1Hr 30 mg/kg vs veh delta: 0, 1, 2, 3 and 5Hr
						Time	10, 130	3.801	0.0002	***		
						Dose x Time	30, 390	5.927	<0.0001	****		
17b	Aged	Xanomeline effects on wake delta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	1.391	0.2613	ns	13	3 mg/kg vs veh delta: 4Hr 10 mg/kg vs veh delta: 1Hr 30 mg/kg vs veh delta: 0, 2, 3, 4 and 5Hr
						Time	10, 120	3.640	0.0003	***		
						Dose x Time	30, 360	7.420	<0.0001	****		
17c	Young	Xanomeline effects on wake theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.081	0.0384	**	14	3 mg/kg vs veh theta: -1, 0 and 5Hr 10 mg/kg vs veh theta: 0 and 3Hr 30 mg/kg vs veh theta: 0 and 1Hr
						Time	10, 130	3.168	0.0012	*		
						Dose x Time	30, 390	13.44	<0.0001	****		
17d	Aged	Xanomeline effects on wake theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	3.008	0.0428	*	13	10 mg/kg vs veh theta: 0 and 1Hr 30 mg/kg vs veh theta: 0, 1, 2, 3,4, 5 and 7Hr
						Time	10, 120	10.76	<0.0001	****		
						Dose x Time	30, 360	28.30	<0.0001	****		
17e	Young	Xanomeline effects on wake alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	2.141	0.1106	ns	14	3 mg/kg vs veh alpha: 0, 1, 2, 3, 5 and 8Hr 10 mg/kg vs veh alpha: 0, 4 and 6Hr 30 mg/kg vs veh alpha: -2, 0, 3 and 5Hr
						Time	10, 130	13.05	<0.0001	****		
						Dose x Time	30, 390	14.72	<0.0001	****		
17f	Aged	Xanomeline effects on wake alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	3.146	0.0368	*	13	3 mg/kg vs veh alpha: 2, 5, 7 and 8Hr 10 mg/kg vs veh alpha: 0 and 1Hr 30 mg/kg vs veh alpha: 0, 1, 2, 5, 6, 7 and 8Hr
						Time	10, 120	14.24	<0.0001	****		
						Dose x Time	30, 360	15.46	<0.0001	****		
17g	Young	Xanomeline effects on wake beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.630	0.0073	**	14	3 mg/kg vs veh beta: 8Hr 10 mg/kg vs veh beta: 0, 3, 5, 6 and 8Hr 30 mg/kg vs veh beta: 0, 1, 6 and 8Hr
						Time	10, 130	15.18	<0.0001	****		
						Dose x Time	30, 390	2.122	0.0007	***		
17h	Aged	Xanomeline effects on wake beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 36	0.2588	0.8545	ns	13	30 mg/kg vs veh beta: 0 and 1Hr
						Time	10, 120	5.445	<0.0001	****		
						Dose x Time	30, 360	8.285	<0.0001	****		
18a	Young	Xanomeline effects on NREM theta power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	11.59	0.0003	***	13-14	3 mg/kg vs veh theta: 0Hr 10 mg/kg vs veh theta: 0Hr
						Time	2, 26	18.49	<0.0001	****		
						Dose x Time	4, 51	21.72	<0.0001	****		
	Young	Xanomeline effects on NREM theta power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	4.216	0.0112	*	13-14	3 mg/kg vs veh theta: 1Hr 10 mg/kg vs veh theta: 1Hr 30 mg/kg vs veh theta: 1and 2Hr
						Time	7, 91	6.504	<0.0001	****		
						Dose x Time	21, 272	4.18	<0.0001	****		
18b	Aged	Xanomeline effects on NREM theta power (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 24	27.85	<0.0001	****	9-13	3 mg/kg vs veh theta: 0Hr 10 mg/kg vs veh theta: 0 and 1Hr
						Time	3, 36	8.425	0.0002	***		
						Dose x Time	6, 69	20.56	<0.0001	****		
	Aged	Xanomeline effects on NREM theta power (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 36	5.430	0.0035	**	7-13	30 mg/kg vs veh theta: 2, 3, 4, 5 and 6Hr
						Time	6, 72	3.549	0.0039	**		
						Dose x Time	18, 210	1.233	0.2373	ns		
18c	Young	Xanomeline effects on NREM alpha power (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	6.957	0.0038	**	13-14	10 mg/kg vs veh alpha: 0Hr
						Time	2, 26	37.30	<0.0001	****		
						Dose x Time	4, 51	9.628	<0.0001	****		
	Young	Xanomeline effects on NREM alpha power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	2.250	0.0977	ns	13-14	30 mg/kg veh alpha: 1 and 2Hr
						Time	7, 91	103.4	<0.0001	****		
						Dose x Time	21, 272	19.98	<0.0001	****		
18d	Aged	Xanomeline effects on NREM alpha power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 24	20.29	<0.0001	****	9-13	10 mg/kg vs veh alpha: 0 and 1Hr
						Time	3, 36	19.88	<0.0001	****		
						Dose x Time	6, 69	13.70	<0.0001	****		
	Aged	Xanomeline effects on NREM alpha power (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 36	10.39	<0.0001	****	7-13	30 mg/kg vs veh alpha: 2, 3 and 4Hr
						Time	6, 72	71.75	<0.0001	****		
						Dose x Time	18, 210	25.01	<0.0001	****		
18e	Young	Xanomeline effects on NREM beta power (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	1.175	0.3246	***	13-14	10 mg/kg vs veh beta: 0Hr
						Time	2, 26	9.909	0.0006	ns		
						Dose x Time	4, 51	3.036	0.0254	*		
	Young	Xanomeline effects on NREM beta power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	9.51	0.0444	*	13-14	3 mg/kg vs veh beta: 1Hr 30 mg/kg veh beta: 4, 5, 6, 7 and 8Hr
						Time	7, 91	56.03	<0.0001	****		
						Dose x Time	21, 272	2.957	<0.0001	****		
18f	Aged	Xanomeline effects on NREM beta power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 24	0.2066	0.8147	ns	9-13	10 mg/kg vs veh beta: 0Hr
						Time	3, 36	4.316	0.0107	*		
						Dose x Time	6, 68	4.010	0.0017	**		
	Aged	Xanomeline effects on NREM beta power (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 36	0.1018	0.9585	ns	7-13	N/A
						Time	6, 72	23.36	<0.0001	****		
						Dose x Time	18, 210	1.049	0.4065	ns		

18g	Young	Xanomeline effects on NREM gamma power (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 26	5.090	0.0136	*	13-14	10 mg/kg vs veh gamma: 0Hr
						Time	2, 26	13.56	<0.0001	****		
						Dose x Time	4, 51	12.44	<0.0001	****		
	Young	Xanomeline effects on NREM gamma power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	5.058	0.0047	**	13-14	3 mg/kg vs veh gamma: 1Hr 30 mg/kg vs veh gamma: 1Hr
						Time	7, 91	9.402	<0.0001	****		
						Dose x Time	21, 272	12.16	<0.0001	****		
18h	Aged	Xanomeline effects on NREM gamma power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	2, 24	27.98	<0.0001	****	9-13	10 mg/kg vs veh gamma: 0 and 1Hr
						Time	3, 36	23.16	<0.0001	****		
						Dose x Time	6, 68	25.53	<0.0001	****		
	Aged	Xanomeline effects on NREM gamma power (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 36	9.237	0.0001	***	7-13	30 mg/kg vs veh gamma: 2 and 3Hr
						Time	6, 72	8.416	<0.0001	****		
						Dose x Time	18, 210	15.13	<0.0001	****		
19a	Young	Donepezil effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	6.746	0.0002	***	13	1mg/kg vs Veh Freq: 2-4, 8-9, 45-52, 54-79Hz 3 mg/kg vs Veh Freq: 2-4, 8-9, 36, 38, 41-79Hz
						Frequency	79, 948	6.279	<0.0001	****		
						Dose x Frequency	316, 3792	5.442	<0.0001	****		
19b	Young	Donepezil effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	2.958	0.0290	*	10-13	0.1mg/kg vs Veh Freq: 62, 71and 79Hz 0.3 mg/kg vs Freq: 77Hz 1mg/kg vs Veh Freq: 61, 68-72, 74 and 76-79Hz 3 mg/kg vs Veh Freq: 0.5-2, 4-5 and 12-13Hz
						Frequency	79, 948	4.501	<0.0001	****		
						Dose x Frequency	316, 3552	1.840	<0.0001	****		
19c	Young	Donepezil effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 36	0.2695	0.8469	ns	9-13	N/A
						Frequency	79, 948	1.448	0.0082	**		
						Dose x Frequency	237, 2284	0.6406	>0.9999	ns		
19d	Young	Donepezil effects on wake gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	2.284	0.0739	ns	13	0.3 mg/kg vs Veh Gamma Power: Time relative to dose: 5Hr 1mg/kg vs Veh Gamma Power: Time relative to dose: -2, 0, 1 2, 3, 5, 7Hr 3 mg/kg vs Veh Gamma Power: Time relative to dose: 1, 3Hr
						Time	10, 120	25.25	<0.0001	****		
						Dose x Time	40, 480	1.896	0.0011	**		
19e	Young	Donepezil effects on NREM delta (SWA)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	0.7516	0.5619	ns	13	3 mg/kg vs Veh SWA: Time relative to dose: 0, 3Hr
						Time	10, 120	126.6	<0.0001	****		
						Dose x Time	40, 467	7.097	<0.0001	****		
19f	Aged	Donepezil effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	5.730	0.0007	***	14	0.1mg/kg vs Veh Freq: 30 Hz 0.3 mg/kg vs Freq: 25-26, 28-30, 56Hz 3 mg/kg vs Veh Freq: 0.5, 2, 6-8, 17-25, 31-50Hz
						Frequency	79, 1027	7.652	<0.0001	****		
						Dose x Frequency	316, 4108	3.180	<0.0001	****		
19g	Aged	Donepezil effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	14.29	<0.0001	****	12-14	3 mg/kg vs Veh Freq: 0.5-2, 21, 25, 27-79Hz
						Frequency	79, 1027	16.22	<0.0001	****		
						Dose x Frequency	316, 3948	9.889	<0.0001	****		
19h	Aged	Donepezil effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.1219	0.9466	ns	11-14	NA
						Frequency	79, 1027	1.571	0.0015	**		
						Dose x Frequency	237, 2601	1.007	0.4598	ns		
19i	Aged	Donepezil effects on wake gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.118	0.0917	ns	14	0.3 mg/kg vs Veh Gamma Power: Time relative to dose: 4Hr 3 mg/kg vs Veh Gamma Power: Time relative to dose: 0, 1 3Hr
						Time	10, 130	24.04	<0.0001	****		
						Dose x Time	40, 520	2.784	<0.0001	****		
19j	Aged	Donepezil effects on NREM delta (SWA) (-2 to 1hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3 ,39	1.017	0.3954	ns	13-14	N/A
						Time	2, 26	3.993	0.0307	*		
						Dose x Time	6, 77	0.8026	0.5710	ns		
	Aged	Donepezil effects on NREM delta (SWA) (2 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	2.711	0.0399	*	12-14	3 mg/kg vs Veh SWA: Time relative to dose: 2, 3Hr
						Time	7, 91	22.97	<0.0001	****		
						Dose x Time	28, 361	4.757	<0.0001	****		
20a	Young	Donepezil effects on wake delta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	2.738	0.0393	*	13	0.3 mg/kg vs veh delta: 2Hr 1mg/kg vs veh delta: 0, 1 2, 5, 6 and 8Hr 3 mg/kg vs veh delta: 2Hr
						Time	10, 120	4.066	<0.0001	****		
						Dose x Time	40, 480	1.159	0.2384	ns		
20b	Aged	Donepezil effects on wake delta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	0.3765	0.8243	ns	14	0.3 mg/kg vs veh delta: 4Hr 1mg/kg vs veh delta: 3Hr 3 mg/kg vs veh delta: 0 and 3Hr
						Time	10, 130	6.110	<0.0001	****		
						Dose x Time	40, 520	2.098	0.0002	***		
20c	Young	Donepezil effects on wake theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	0.7145	0.5861	ns	13	0.1mg/kg vs veh theta: 0 and 3Hr 0.3 mg/kg vs veh theta: 5 and 7Hr 1mg/kg vs veh theta: 3 and 7Hr 3 mg/kg vs veh theta: 0, 3, 5, 7 and 8Hr
						Time	10, 120	6.483	<0.0001	****		
						Dose x Time	40, 480	3.186	<0.0001	****		
20d	Aged	Donepezil effects on wake theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	0.3562	0.8385	ns	14	3 mg/kg vs veh theta: 0 and 1Hr
						Time	10, 130	5.046	<0.0001	****		
						Dose x Time	40, 520	5.441	<0.0001	****		
20e	Young	Donepezil effects on wake alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	1.208	0.3196	ns	13	0.3 mg/kg vs veh alpha: 2 and 4Hr 1mg/kg vs veh alpha: 2Hr 3 mg/kg vs veh alpha: 0Hr
						Time	10, 120	12.34	<0.0001	****		
						Dose x Time	40, 480	2.196	<0.0001	****		

20f	Aged	Donepezil effects on wake alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	1.022	0.4047	ns	14	1mg/kg vs veh alpha: 7Hr 3 mg/kg vs veh alpha: 0Hr
						Time	10, 130	7.530	<0.0001	****		
						Dose x Time	40, 520	5.162	<0.0001	****		
20g	Young	Donepezil effects on wake beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	2.526	0.0528	ns	13	N/A
						Time	10, 120	16.76	<0.0001	****		
						Dose x Time	40, 480	1.394	0.0593	ns		
20h	Aged	Donepezil effects on wake beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	2.946	0.0286	*	14	0.3mg/kg vs veh beta: 2 and 5Hr 3 mg/kg vs veh beta: 0, 1, 2 and 7Hr
						Time	10, 130	12.19	<0.0001	****		
						Dose x Time	40, 520	7.358	<0.0001	****		
21a	Young	Donepezil effects on NREM theta power	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	1.911	0.1238	ns	10-13	0.1mg/kg vs veh theta: 6 and 8Hr 3 mg/kg vs veh theta: 0 and 2Hr
						Time	10, 120	74.20	<0.0001	****		
						Dose x Time	40, 476	5.319	<0.0001	****		
21b	Aged	Donepezil effects on NREM theta power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.390	0.0002	***	13-14	N/A
						Time	2, 26	12.07	0.2602	ns		
						Dose x Time	6, 77	0.5670	0.7553	ns		
	Aged	Donepezil effects on NREM theta power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	0.4093	0.8011	ns	12-14	3 mg/kg vs veh theta: 1Hr
						Time	7, 91	19.27	<0.0001	****		
						Dose x Time	28, 361	2.146	0.0008	***		
21c	Young	Donepezil effects on NREM alpha power	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	1.828	0.1388	ns	10-13	1mg/kg vs veh alpha: 6Hr 3 mg/kg vs veh alpha: 2Hr
						Time	10, 120	63.06	<0.0001	****		
						Dose x Time	40, 476	1.532	0.0222	*		
21d	Aged	Donepezil effects on NREM alpha power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.503	0.2289	ns	13-14	N/A
						Time	2, 26	10.49	0.0005	***		
						Dose x Time	6, 77	0.2202	0.9692	ns		
	Aged	Donepezil effects on NREM alpha power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	4.400	0.0039	**	12-14	3 mg/kg vs veh alpha: 1, 2, 3 and 4Hr
						Time	7, 91	96.11	<0.0001	****		
						Dose x Time	28, 361	68.04	<0.0001	****		
21e	Young	Donepezil effects on NREM beta power	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	1.255	0.3006	ns	10-13	0.1mg/kg vs veh beta: 6Hr 1mg/kg vs veh beta: 8Hr 3 mg/kg vs veh beta: 0 and 2Hr
						Time	10, 120	87.41	<0.0001	****		
						Dose x Time	40, 476	3.789	<0.0001	****		
21f	Aged	Donepezil effects on NREM beta power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.316	0.2831	ns	13-14	N/A
						Time	2, 26	3.979	0.0311	*		
						Dose x Time	6, 77	0.4358	0.8528	ns		
	Aged	Donepezil effects on NREM beta power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	1.851	0.1332	ns	12-14	3 mg/kg vs veh beta: 1Hr
						Time	7, 91	20.31	<0.0001	****		
						Dose x Time	28, 361	4.372	<0.0001	****		
21g	Young	Donepezil effects on NREM gamma power	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	1.053	0.3902	ns	10-13	0.3 mg/kg vs veh beta: 8Hr 1mg/kg vs veh beta: 8Hr 3 mg/kg vs veh beta: 0Hr
						Time	10, 120	26.94	<0.0001	****		
						Dose x Time	40, 476	8.291	<0.0001	****		
21h	Aged	Donepezil effects on NREM gamma power (-2 to 0hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.5374	0.0252	*	13-14	N/A
						Time	2, 26	4.253	0.6295	ns		
						Dose x Time	6, 77	0.3440	0.9170	ns		
	Aged	Donepezil effects on NREM gamma power (1 to 8hr)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	9.151	<0.0001	****	12-14	3 mg/kg vs veh gamma: 1and 2Hr
						Time	7, 91	8.717	<0.0001	****		
						Dose x Time	28, 361	11.75	<0.0001	****		

Table 2.4. Detailed statistical analysis.

	Vehicle				30 mg/kg xanomeline				Vehicle				3 mg/kg donepezil			
Time (minutes)	30	60	120	240	30	60	120	240	30	60	120	240	30	60	120	240
Autonomic Nervous System																
Ptosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exophtalmus	0	0	0	0	0.166667	0	0	0	0	0	0	0	0	0	0	0
Corneal reflex loss	0.167	0	0	0	0.833	0.500	0.333	0.333	0	0	0	0	0	0	0	0
Pinna reflex loss	0	0	0	0	0.333333	0.333	0	0	0	0	0	0	0	0	0	0
Piloerection	0	0	0	0	0.167	0.500	0.167	0	0	0	0	0	0.500	0.333	0.333	0
Respiratory rate	0	0	0	0	1.500	1.167	0.333	0	0	0	0	0	0.333	0.333	0	0
Writing	0	0	0	0	0	0	0	0	0	0	0	0	0.167	0.167	0	0
Tail erection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lacrimation	0	0	0	0	0	0.166667	0.333	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	1.500	0.500	0.333	0	0	0	0	0	0	0	0	0
Vasodilation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Skin color	0	0	0	0	0.333	0.333	0	0	0	0	0	0	0	0	0	0
Irritability	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baseline pupil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pupil reaction	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Somatomotor Systems																
Motor activity	0	0	0	0	1.333	1.333	0.333	0	0	0	0	0	0.500	0.333	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arch/Roll	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0.833	0.333	0	0	0	0	0	0	0.667	0.333	0	0
Leg weakness	0	0	0	0	0.667	0.500	0	0	0	0	0	0	0.333	0.167	0	0
Rigid stance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spraddle	0	0	0	0	0.500	0.333	0	0	0	0	0	0	0	0	0	0
Placing loss	0	0	0	0	1.000	0	0	0	0	0	0	0	0	0	0	0
Grasping loss	0	0	0	0	0	0	0	0	0	0	0	0	0.167	0	0	0
Righting loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Catalepsy	0.333	0	0	0	2.000	1.333	0.333	0	0	0	0	0	0	1.167	0.333	0.333
Tail pinch	0	0	0	0	0.2	0	0	0	0	0	0	0	0.333	0	0	0
Escape loss	0	0.167	0	0	1.500	1.667	0.667	0.0	0	0.167	0	0.333	0.667	0.667	0.833	0.333
	Dose $F_{(1, 10)}$=28.07, p=0.0003, Time x Dose $F_{(3, 30)}$=17.75, p<0.0001, Time $F_{(3, 30)}$=20.31, p<0.0001 Post hoc analysis: 30 mg/kg xanomeline vs vehicle: 30 min, p<0.0001; 60 min, p<0.0001								Dose $F_{(1, 10)}$=5.169, p=0.0463, Time x Dose $F_{(3, 30)}$=1.367, p=0.2718, Time $F_{(3, 30)}$=1.100, p=0.3643 Post hoc analysis: No significant difference at any time point							
	For all behaviors scored: 0 = normal, 1= mild effect and 2 = severe effect															

Table 2.5: Xanomeline and donepezil produce adverse side effects on the Modified Irwin testing battery in non-pathologically aged mice during the active phase.

	Vehicle				30 mg/kg xanomeline				Vehicle				3 mg/kg donepezil			
Time (minutes)	30	60	120	240	30	60	120	240	30	60	120	240	30	60	120	240
Autonomic Nervous System																
Ptosis	0	0	0	0	0	0.167	0.333	0	0	0	0	0	0	0	0	0
Exophtalmus	0.167	0	0	0	0.167	0	0	0	0	0	0	0	0	0	0	0
Corneal reflex loss	0	0	0	0	0.500	0.333	0.500	0	0	0	0	0	0	0	0	0
Pinna reflex loss	0	0	0	0	0	0.333	0	0	0	0	0	0	0.833	1.000	1.000	0.333
Piloerection	0	0	0	0	0.500	0.833	0.500	0	0	0	0	0	0.833	0.500	0.333	0
Respiratory rate	0	0	0	0	1.667	1.500	0.833	0	0	0	0	0	1.167	1.000	0.333	0
Writing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tail erection	0	0	0	0	0	0	0	0	0	0	0	0	0	0.167	0.167	0
Lacrimation	0	0	0	0	0.167	0.167	0.500	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	1.333	0.833	0.167	0	0	0	0	0	0	0	0	0
Vasodilation	0	0	0	0	0.333	0	0	0	0	0	0	0	0	0	0	0
Skin color	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Irritability	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baseline pupil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pupil reaction	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Somatomotor Systems																
Motor activity	0	0	0	0	1.500	1.167	0.333	0	0	0	0	0	1.500	1.167	0.833	0.167
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arch/Roll	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0.833	1.000	0.333	0	0	0	0	0	1.500	1.167	0.833	0.167
Leg w eakness	0	0	0	0	0.833	0.667	0.333	0	0	0	0	0	0.333	0.333	0	0
Rigid stance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spraddle	0	0	0	0	1.000	1.500	0	0	0	0	0	0	0.333	0.333	0.167	0
Placing loss	0	0	0	0	1.333	0	0	0	0	0	0	0	0	0	0	0
Grasping loss	0	0	0	0	0.0	0	0	0	0	0	0	0	0	0.167	0	0
Righting loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Catalepsy	0	0	0	0	1.667	1.333	0	0	0	0	0	0	1.500	1.333	0.333	0.333
Tail pinch	0	0	0	0	0.333	0.333	0	0	0	0	0	0	0	0	0	0
Escape loss	0	0	0	0.167	2.000	1.833	1.000	1.000	0.167	0.167	0.167	0.667	1.000	1.000	1.333	0.333
	Dose $F_{(1, 10)}$=33.23, p=0.0002, Time x Dose $F_{(3, 30)}$=21.12, p<0.0001, Time $F_{(3, 30)}$=21.02, p<0.0001 Post hoc analysis: 30 mg/kg xanomeline vs vehicle: 30 min, p<0.0001; 60 min, p<0.0001; 120 min p=0.0408								Dose $F_{(1, 10)}$=20.79, p=0.0010, Time x Dose $F_{(3, 30)}$=8.442, p=0.0003, Time $F_{(3, 30)}$=6.759, p=0.0013 Post hoc analysis: 30 mg/kg xanomeline vs vehicle: 30 min, p<0.0001; 60 min, p<0.0001; 120 min p=0.0270							
	For all behaviors scored: 0 = normal, 1= mild effect and 2 = severe effect															

Table 2.6: Xanomeline and donepezil produce adverse side effects on the Modified Irwin testing battery in non-pathologically aged mice during the inactive phase.

CHAPTER 3

The M_1 Muscarinic Acetylcholine Receptor Positive Allosteric Modulator VU0453595 Normalizes Sleep Disturbances in Aged mice and Enhances Arousal in Rodents and Non-Human Primates

3.1. Introduction

Declining integrity of the central cholinergic system is associated with disruptions in sleep/wake architecture, arousal, and cognition in non-pathological aging and neurodegenerative disease (Lloret et al., 2020; Prinz, Peskind, et al., 1982). Alterations in multiple synaptic markers of basal forebrain cholinergic structure and function, have been reported in aging and Alzheimer's disease (AD) patient populations (Aghourian et al., 2017; Bartus et al., 1982; Dumas and Newhouse, 2011; Terry and Buccafusco, 2003). Additionally, degeneration of the basal forebrain cholinergic projection system is a robust and reliable predictor of entorhinal and neocortical neurodegeneration and constitutes an early event in the development of AD (Fernández-Cabello et al., 2020). Specifically, decreases in the cortical expression of cholinergic markers have been correlated with age- and/or neurodegenerative disease-related impairments in attention, memory, and executive functions (Drachman and Leavitt, 1974; Dumas and Newhouse, 2011; M. Mesulam, 2004; Richter et al., 2014; Schliebs and Arendt, 2006). Similar deteriorations in cortical cholinergic integrity and cognitive performance have been well documented across aged rodent and nonhuman primate (NHP) species (Voytko et al., 2001; C. F. Wu et al., 1988). The impact of deteriorating central cholinergic circuitry has also been linked with abnormalities in sleep/wake architecture and arousal, which are thought to directly contribute to and exacerbate the cognitive impairments observed in individuals with advanced age and dementia (Lloret et al., 2020; Prinz, Peskind, et al., 1982). Accumulating evidence indicates that disruptions in sleep represent a

significant risk factor for AD, with older dementia patients exhibiting shorter sleep duration and fragmented sleep, elevated rates of sleep disordered breathing and altered circadian rest/activity patterns. Collectively, these findings have led to therapeutic approaches for the enhancement of central cholinergic signaling to ameliorate symptoms associated with pathologic changes in aging and neurodegenerative diseases such as AD (Bubu et al., 2017).

To date, acetylcholinesterase inhibitors (AChEIs) represent the only FDA-approved treatment for the cognitive impairments associated with AD that specifically target the cholinergic system. AChEIs block the degradation of acetylcholine (ACh) resulting in increased synaptic levels of ACh (Dumas and Newhouse, 2011). While AChEIs produce modest therapeutic effects on cognitive impairments during the early stages of AD, these drugs are associated with dose-limiting adverse effects due to nonselective activation of central and peripheral muscarinic acetylcholine receptors (mAChRs) (Galimberti and Scarpini, 2016). Of the five different mAChR subtypes activated by ACh (M_1-M_5), M_1 is highly expressed postsynaptically in brain regions that regulate arousal, sleep and cognition, including the cortex, striatum and hippocampus (Levey et al., 1991, 1995; Marino et al., 1998; Rouse et al., 1998, 1999). Thus, activation of M_1-mAChRs was thought to be a promising strategy for the symptomatic treatment of AD-related cognitive deficits. Early clinical studies with xanomeline, an M_1/M_4-mAChR subtype-preferring orthosteric agonist, showed significant efficacy in treating AD-related behavioral disturbances and trends towards improving reaction time and verbal memory deficits (Bodick et al., 1997). Xanomeline and other M_1-preferring orthosteric agonists also produced pro-cognitive effects in rodents and NHPs (C. K. Jones et al., 2012), yet have failed in clinical development due to off-target activation of peripheral mAChRs similar to those observed with AChEIs (Bodick et al., 1997).

Using an alternative strategy for the development of subtype-selective activators of M_1, our group and others have focused on identification of ligands that target less highly conserved regions of the receptor, termed allosteric sites, which are distinct from the highly conserved ACh binding site. This approach has resulted in the discovery of multiple M_1 positive allosteric

modulators (PAMs), including VU0453595, with greater than 10-fold selectivity for M_1 over the other mAChR subtypes and suitable pharmacokinetic properties for dosing in rodent and non-human primate (NHP) species (Bubser et al., 2012; Conn, Lindsley, et al., 2009; C. K. Jones et al., 2012). VU0453595 does not directly activate M_1, but potentiates the effects of presynaptically released ACh, thereby maintaining the spatial and temporal pattern of endogenous cholinergic signaling (Conn, Jones, et al., 2009). Previous studies have shown that VU0453595, like other M_1 mAChR PAMs, enhances cognitive performance without dose-limiting adverse effects in rodents (Ghoshal et al., 2016; Gould et al., 2015; Grannan et al., 2016; Lv et al., 2017; L. Ma et al., 2009; Moran et al., 2018; Rook et al., 2018; Uslaner et al., 2013). More recently, our group reported that the investigational drug candidate VU319, a highly optimized M_1 PAM, was well tolerated in a Phase I single ascending dose clinical study with dose-related target engagement including improved reaction times and increased event-related potential amplitudes in an incidental memory task (Conley et al., 2019; P. Newhouse et al., 2019).

While accumulating evidence supports the further development of M_1 PAMs for cognitive decline associated with AD, there is limited information on the impact of selective activation of M_1 on sleep/wake architecture and arousal during non-pathological aging. The central cholinergic system plays a critical role in the regulation of normal sleep-wake patterns across species. ACh levels are highest in the morning during peak wakefulness, then decrease throughout the day to lowest levels during the early stages of non-rapid eye movement (NREM) sleep, followed by subsequent increases with the transition from NREM to the later stages of rapid eye movement (REM) sleep (Brown et al., 2012). Increases in ACh stimulate higher frequency electroencephalography (EEG) activity consistent with wakefulness and REM sleep (Brown et al., 2012; Graef et al., 2011; Platt and Riedel, 2011). Previous studies in young adult humans or NHPs have reported that M_1 PAMs alter arousal by decreasing power in low frequency ranges, i.e., shifting power from low to high frequency ranges (Uslaner et al., 2018), or by attenuating the

increase in lower frequency power elicited by the nonselective mAChR antagonist scopolamine in NHPs (Kurimoto et al., 2019).

The present study is the first systematic evaluation of the effects of the M_1 mAChR PAM VU0453595 on sleep/wake architecture and arousal using EEG in young rats, mice, and NHPs and in normally aged mice. Additionally, these studies examined the therapeutic index between doses that modulate sleep/wake architecture and/or arousal relative to dose-limiting adverse effects in comparison with the AChEI donepezil and xanomeline. These data provide a critical foundation for future studies of M_1 PAMS in preclinical dementia models and AD patients.

3.2. Methods

Subjects.

Young adult male Sprague-Dawley rats (n=12, 250-275g; Envigo, Indianapolis, IN). Young (4-6 month; n=13) or aged (22-26 month; n=10) adult male wild-type mice or young adult male M_1 KO mice (4-6 months; n=6) with the same genetic background (C57BL/6NTac; Taconic) and drug-naïve (n=8) young adult (4-8 yr old at start of study), male cynomolgus macaques (Macaca fascicularis) served as subjects. All animals were socially housed prior to surgery.

Animals lived in a temperature and humidity-controlled environment under a 12/12 h light-dark cycle with water available ad libitum. Rodents had ad libitum access to food in their home cages. NHPs were weighed weekly and fed enough food daily (Purina LabDiet 5045, St Louis, MO, USA and fresh fruit and vegetables) to maintain healthy body weights and appearance as determined by daily inspection and periodic veterinary examinations. All animals were individually housed following implantation of EEG devices. All experiments were approved by the Vanderbilt University (mice/rats) or Wake Forest School of Medicine (NHPs) Animal Care and Use Committees, and experimental procedures conformed to guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. Environmental enrichment

was provided as outlined in the Animal Care and Use Committee of Wake Forest University Non-Human Primate Environmental Enrichment Plan.

Compounds.

VU0453595, BQCA, and xanomeline L-tartrate were synthesized in-house (Ghoshal et al., 2016; Shirey et al., 2009); donepezil was obtained from Sigma Aldrich (St. Louis, MO). VU0453595 and BQCA were formulated as a microsuspension in 5% and 20% beta-cyclodextrin, respectively, in sterile water. Donepezil and xanomeline were formulated in sterile saline and water, respectively, as aqueous solutions. All compounds' formulations were adjusted to pH 6-7. Compounds were administered at 10 mL/kg (mice) intraperitoneally (i.p.) and 2 mL/kg (rats) i.p except for BQCA (administered subcutaneously [s.c]). For NHPs, VU0453595 was administered orally (i.g.) via a nasogastric tube at 5 mg/mL (3.0 and 10 mg/kg) or 10 mg/mL (30 mg/kg). Xanomeline (1.0, 3.0 mg/kg) was administered s.c. at 0.1 mL/kg. Donepezil (3.0, 10 mg/kg) was administered orally as a powder mixed in a palatable treat and hand-fed to each NHP (EEG) or via nasogastric tube in saline (pharmacokinetic studies). The dose ranges tested have previously shown to increase cognitive performance in rodents (Gould et al., 2015; Lv et al., 2017; Moran et al., 2018) or reverse pharmacological disruptions in rodent or NHPs (Ghoshal et al., 2016; Vardigan et al., 2015). Administration of each compound followed a within-subject, counter-balanced design such that each animal received all doses with a minimum of 5 days (washout) between doses; separate vehicle determinations were conducted for each compound.

Electroencephalography

Surgery

For telemetry studies, animals were surgically implanted under isoflurane anesthesia with a telemetric transmitter (mouse, F20-EET; rat, 4-ET; NHP, D70-EEE; Data Sciences International [DSI], Minneapolis, MN) for recording EEG, electromyographic (EMG), and motor activity as previously described Gould et al., 2016; Nedelcovych et al., 2015; Rook et al., 2015). Transmitters were implanted subcutaneously just off the midline of the dorsal flank of each animal (mouse, rat)

or intraperitoneally (NHP) under aseptic conditions. Transmitter leads were tunneled subcutaneously to the skull. Holes were drilled in the skull and exposed wires were placed directly in contact with the dura and secured via dental cement (Butler Schein, USA). For mice, one set of leads were implanted (+1 mm, and -3 mm from Bregma, respectively and ± 2 mm contralateral to the midline). For rats, three sets of leads were placed bilaterally 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). For NHPs, EEG lead placements were selected based on the International 10-20 System of Electrode Placement. Two sets of leads were placed bilaterally corresponding with F3-F4 (frontal cortex) and P3-P4 (parietal cortex) placements. For NHP surgeries, screws were placed into the drilled holes in the skull and wires were wrapped around the screws. In all animals, an additional set of leads were placed bilaterally in the nuchal muscle for EMG recording. NHPs received no other drugs besides pharmacological challenges (see below), with the exception of infrequent (less than once per month) exposure to ketamine used as an anesthetic to facilitate veterinary procedures. Following surgery, animals were individually housed.

Examining *sleep/wake architecture and qEEG*

For all studies, EEG and EMG were recorded from the home cage of each animal continuously for 24 hrs beginning at the onset of the light cycle on the day of each study. Telemetric EEG and EMG waveform data were collected using Dataquest A.R.T. software (DSI). Based on limitation of the transmitters, data were continuously sampled at a rate of 100 Hz (mouse, NHP) or 500 Hz (rat) and transmitted via a receiver (RPC-1, mouse, rat; RMC-1, NHP; DSI) placed below the cage of each mouse/rat or on the side of the cage of each NHP. Each receiver is connected to a data exchange matrix (DSI, MN), which transfers data to a computer for offline analysis. This study design allowed us to assess wake-promoting and sleep-altering effects in rodents during the time period they predominately sleep, and to assess effects on

arousal in NHPs (since NHPs rarely sleep during the light period, effects on sleep were not direclty examined).

Sleep Staging and analysis

Trained observers, blinded to condition (age, genotype or pharmacological challenge) scored each 10-second epoch (rats, NHPs) or 5-second epochs (mice) using Neuroscore 3.0 software (DSI) to determine sleep/wake stages, including wake, non-rapid eye movement (NREM) or rapid eye movement (REM) sleep based on accepted characteristic oscillatory patterns as previously published by our group (Gould et al., 2016; Nedelcovych et al., 2015; Rook et al., 2015). The amount of time in each stage (wake, NREM, REM) in 1-hr (rat, NHPs) or 2-hr (mice) bins across a 24-hr period served as primary dependent measures to determine effects of age, genotype or acute pharmacological challenge.

qEEG Spectral Power Analysis

Following sleep staging, quantitative EEG (qEEG) relative power spectra were computed in 1Hz bins from 0.5 to 100 Hz (rat) or 50Hz (mouse, NHP) using a Fast Fourier Transform with a Hamming window and overlap ratio of 0.5. Relative power within each 1Hz increment was subsequently binned by stage (wake, NREM or REM), then averaged across a select time period to yield the state-dependent relative power spectrum for each animal and condition. Differences in spectral power between genotypes or dose-effect determinations were examined in 1-hr bins (mouse, rat) or 4-h bins (NHPs) in a state-dependent (Wake, NREM, REM) manner. (Nedelcovych et al., 2015; Rook et al., 2015). For mice and rats, pharmacological effects on arousal during wake were determined first as within subject changes by expressing the power spectrum 1-2 h post dosing as a percent change within each respective 1 Hz interval from the 1-h interval prior to dosing (baseline). (e.g. [power 1-2 h post dosing/ 1 h baseline x 100]-100). Data from each animal was then averaged. For NHPs, data were analyzed in a similar manner except for the following three exceptions: 1) since dosing occurred early in light period, there was not a sufficient time period to analyze within-subject changes within the same day. As a baseline, relative power

across the spectrum was averaged for the three vehicle-treatment conditions (one per dose-response curve). Effects of donepezil, VU0453595, or xanomeline and their respective vehicle are expressed as a percent change from this averaged baseline; Due to larger variability within the power spectra and lower sample size, data were collapsed 2) across the 4-hr time period following dosing; and 3) into frequency bins as Delta (0.5-4Hz), Theta (4-8 Hz), Alpha (8-13 Hz), Beta (13-30 Hz), Low Gamma (30-50 Hz). For all species, pharmacologically-induced changes in qEEG are discussed in terms of these power bands according to convention (Gould et al., 2016; Nedelcovych et al., 2015).

Assessing effects of M_1 mAChR PAM VU0453595, BQCA, and donepezil on sleep/wake duration and qEEG in young rat.

To examine selective versus nonselective effects of enhancing cholinergic function, VU0453595 (3.0-30 mg/kg, i.p.), BQCA (3.0-30 mg/kg, s.c.) and donepezil (1.0-10 mg/kg, i.p.) or their respective vehicle were administered 2 h after light onset (quiescent period) in young rats. EEG, EMG and activity were monitored continuously for 24 hrs.

Assessing effects of VU0453595, donepezil and xanomeline on sleep/wake and qEEG in young cynomolgus NHP. As a proof of concept study to examine translatability of EEG as a biomarker of CNS function in higher order, gyrencephalic species, VU0453595 (3.0-30 mg/kg, i.g.), donepezil (3.0-10 mg/kg, p.o), and xanomeline (1.0, 3.0 mg/kg, s.c.) were tested in 5 young adult cynomolgus macaques. Thirty minutes after light onset (when arousal levels were presumably low), test compounds were administered.

Assessing unconditioned behavioral effects and plasma concentrations of VU0453595, xanomeline, and donepezil in young cynomolgus NHP.

To establish dose-effect relationships and a relative therapeutic index, we determined plasma concentrations of each compound. To test the hypothesis that M_1 mAChR PAMs elicit less severe adverse effects than nonselective agonists or AChEIs, we implemented a qualitative rating scale to compare the effects of VU0453595, donepezil, and xanomeline in cynomolgus

macaques on cholinergic-mediated changes in somatomotor and autonomic function. (see Table 3.1).

All pharmacokinetic studies were performed in 4 NHPs. Each NHP had been fitted with an aluminum collar (Primate Products, Redwood City, CA, USA) and trained to sit calmly in a primate chair (Primate Products) using a specially designed stainless-steel pole that attached to the collar (Primate Products). NHPs were trained to move from cage to primate restraint chair under minimal duress and habituated to the passing of an infant feeding tube (5 French,1.7 mm X 381 mm) down the nose, through the esophagus, and into the stomach for intragastric (i.g.) intubation for oral delivery of compound and for blood collection procedures (leg presentation for percutaneous stick to the femoral vein). Vehicle, VU0453595 (3.0-30 mg/kg, i.g.), donepezil (3.0-10 mg/kg, i.g.), and xanomeline (1.0, 3.0 mg/kg, s.c.) were administered 30 minutes after light onset. Blood samples were collected from 4 NHPs at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hr post dosing to determine plasma concentrations. Quantitation of plasma concentrations of VU0453595, donepezil, and xanomeline were performed via HPLC-MS/MS essentially as described previously (Bubser et al., 2014; Ghoshal et al., 2016), with the following modifications. A 10 % B gradient was held for 0.2 min and was linearly increased to 90 % B over 1.2 min, held (isocratic) for 0.1 min, and then decreased to 10 % B over 1 min before re-equilibration of the column for 0.4 min (total run time of 2.0 min per sample). Data are presented as mean (n=2-3/dose) concentration-time profiles.

Pharmacokinetic parameters were determined by non-compartmental analysis using WinNonlin v.5.3 (Pharsight Corp., Mountain View, CA). Prior to compound administration and just

Autonomic nervous System	Definition (assessment)
Salivation	Saliva on/around lips/mouth

Lacrimation	Clear fluid from eyes
Urination	Qualitative assessment of urine output in pan beneath chair
Defecation (amount)	Qualitative assessment of fecal output in pan beneath chair
Defecation (consistency)	Consistency of fecal output in pan beneath chair (hard/soft/diarrhea)
Emesis	Presence of vomit
Miosis	Decreased pupil diameter
Mydriasis	Increased pupil diameter
Ptosis	Drooping of upper eyelid
Exophtalmos	Abnormal protrusion (bulging) of eyeball from the orbit
Piloerection	Erection of fur
Respiratory Rate	Increase/decrease in rate of inhalation/exhalation
Penile Erection	Hardening/stiffening of penis
Yawn	Full extension of the jaws exposing teeth/gums
Vasodilation	Dilation of blood vessels; evaluated by observing face (redness/flushing)
Vasoconstriction	Constriction of blood vessels; evaluated by observing face (pale/white)
Irritability	Evaluated by observation at rest and in response to mild stimulation when handled or in response to noise
Body Temperature	Colonic temperature in degrees Celsius
Somatomotor Systems	
Physical Appearance	Overall physical characteristics including coat consistency, skin color, affect
Tremor	Involuntary movements resulting from rapid alternating contraction and relaxation of opposing muscle groups
Leg Weakness	Weakness in muscle tone or resistance when extended by experimenter

Catalepsy	A state of markedly diminished responsiveness in which there is a loss of voluntary motion and a plastic rigidity of the muscles; response is measured in same way as leg weakness
Visuo-Motor Coordination	Determined by presenting a small treat within arm's reach of monkey and assessing ability to reach and retrieve
Posture	Change in position in restraint chair; slumped/stiff/rigid
Unrest	Change in activity/motor output; (e.g. restlessness, fidgeting in chair; frequent re-posturing or change in direction of movement)
Stereotypies	Repeated movements; often abnormal
Arousal	Degree of vigilance ranging from attentive to surrounding stimuli, hypervigilant to external stimuli or inattentive
Sedation	Degree of drowsiness ranging from fully awake to slowed response to stimuli; change in posture to eyes closed or fully asleep
Oral Dyskinesia	Excessive jaw movement; bruxism or tongue protrusions
Bradykinesia	Stiff or slow movements ranging from normal to fixed, sustained posture
Dystonia	Twisting or repetitive abnormal movement of head, neck, torso, limbs, gaping or grimacing
Behaviors and Scale adapted and modified from Irwin 1968, Patel 1997 and Andersen et al 2003	

Table 3.1. NHP Adverse Drug Effect Test Battery. Effects of pharmacological challenges in NHP's will be scored on a scale of 0, 1, or 2; where 0=no effect; normal, 1= a slight effect, and 2= a marked effect. Baseline (pre-drug) assessments are required for determining changes within each animal. Where appropriate, a positive score will be given for increases, and a negative score will be given for decreases, in the item scored.

prior to blood collection at each timepoint, a brief (<5 min) assessment of general health and autonomic/somatomotor function was conducted to assess potential cholinergic-mediated adverse effects. This assessment incorporated aspects of prior batteries examining adverse or off-target effects across species (Andersen et al., 2003; Bubser et al., 2014; Ghoshal et al., 2016; Gould et al., 2016; Patel et al., 1997; Vardigan et al., 2015). Briefly, trained observers examined

each NHP for changes in 18 measures of autonomic function (including functions known to be sensitive to cholinergic stimulation such as salivation, lacrimation, urination, and defecation, temperature), as well as 13 measures of somatomotor function. Ratings were assigned on a scale of 0, 1, or 2; where 0= normal or no change from baseline, 1= a slight effect, and 2= a marked effect. Scores at each timepoint were averaged across all NHPs that received each dose of each compound.

Assessing effects of M_1 mAChR PAM VU0453595 on sleep/wake duration and qEEG in young and aged mouse.

VU0453595 (3.0-30 mg/kg, i.p.) or vehicle were administered 2 h after light onset (quiescent period, when rodents predominately sleep) to young (4-6 months) or aged (22-26 months) wildtype mice or to young (4-6 months) M_1 KO mice (30 mg/kg VU0453595 and vehicle only) to confirm M1 PAM selectivity.

Statistics.

Sleep/wake architecture and qEEG data are presented as means ± S.E.M. and plasma concentrations are shown as means ± S.D. When possible, a repeated measures two-way analysis of variance (ANOVA; matching both factors) was applied. When group sizes were uneven, a repeated measures, mixed effects model (REML) was applied (see Table 3.2 for complete details of tests, factors and results). In all cases, main effects were followed by Dunnett's or Bonferroni's multiple comparison test (see Table 3.2). GraphPad Prism version 8.0 was used for all graphing and statistical applications. For analyses assessing pharmacological effects on sleep and qEEG, both time and treatment dose were used as repeated factors followed by a Dunnett's multiple comparisons test comparing treatment dose to vehicle-treated conditions. When comparing age (young vs. aged) or genotype (wildtype vs. M_1 mAChR KO) effects were performed by comparing the vehicle-treated groups within each respective condition using Bonferroni's test (time was a repeated factor). qEEG data were analyzed across the entire power spectrum (mouse, rat) or across a priori defined power bands (NHPs; Delta (0.5-4Hz), Theta (5-

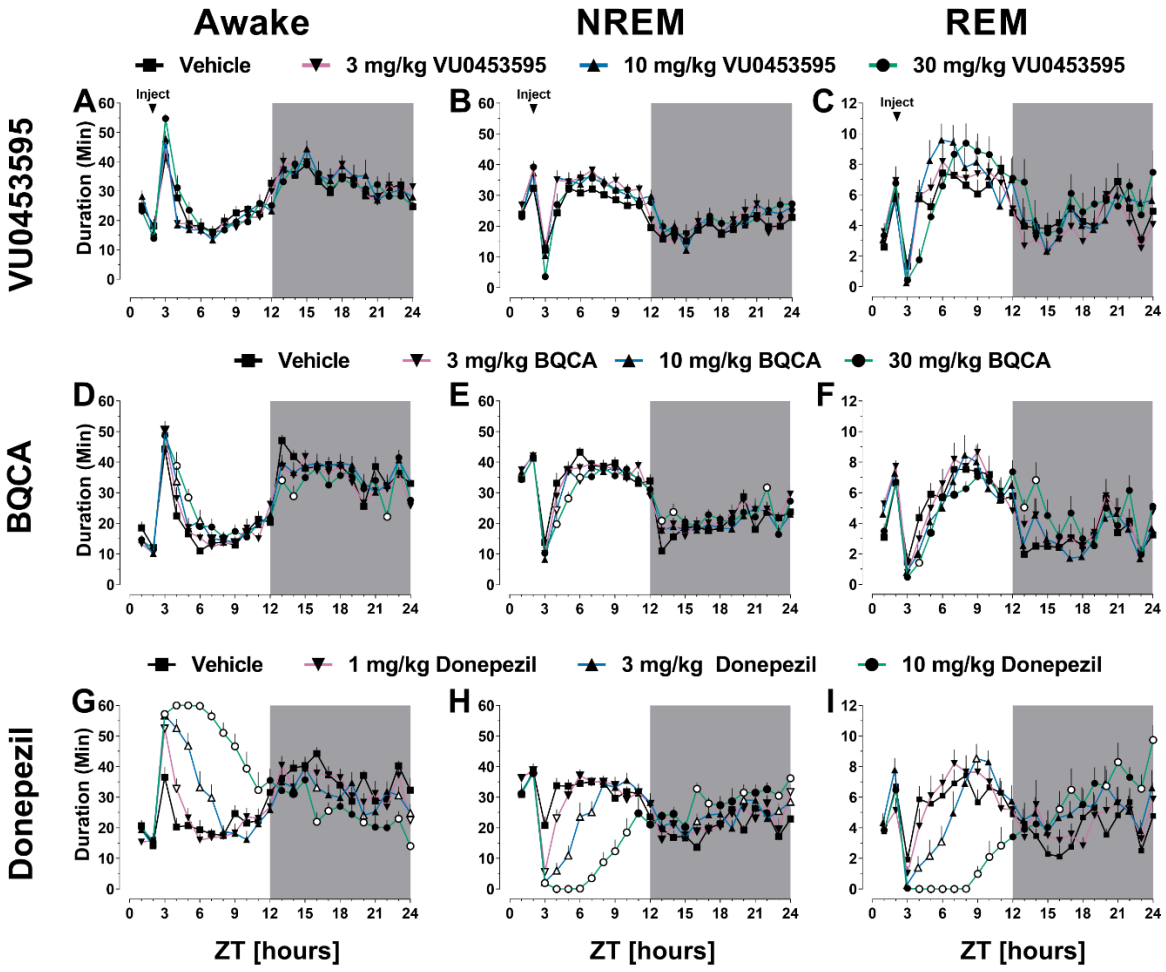

Figure 3.1. The M_1 mAChR PAM VU0453595 did not alter sleep/wake architecture in young adult rats. Shown is the duration of time awake (A, D, G), in non-REM (NREM) sleep (B, E, H), or in REM sleep (C, F, I). Following compound administration 2 hours into the light (inactive) phase (see arrowhead), VU0453595 did not change time awake (A), in non-REM (NREM) sleep (B), or in REM sleep (C). 3 mg/kg BQCA decreased time awake at ZT 13; 10 mg/kg BQCA increased time awake at ZT 4 and 6, and 30 mg/kg BQCA increased time awake at ZT 4 and 5, and decreased time awake at 13, 14, and 22 (D). 10 mg/kg BQCA decreased NREM sleep at ZT 4 and 6; 30 mg/kg BQCA decreased NREM sleep at ZT 4-6, and increased NREM sleep duration at ZT 13,14, and 22 (E). 30 mg/kg BQCA decreased REM sleep time at ZT 4, and increased REM sleep at 13-14 (F). 1.0 mg/kg donepezil increased duration of time awake at the ZT 3,4 and decreased time awake at ZT 24. 3.0 mg/kg donepezil increased time awake at ZT 3-7 and decreased time awake at ZT 16,20,23,24. 10 mg/kg donepezil increased time awake at ZT 3-11, and decreased time awake ZT 16,17, 20, 23, and 24 (G). 1.0 mg/kg donepezil decreased duration of NREM sleep at ZT 3 and 4 time points with an increase at ZT 24; 3.0 mg/kg donepezil decreased duration of NREM sleep at ZT 3-7 and increased NREM sleep duration at ZT 16, 20, and 23.10 mg/kg donepezil decreased NREM duration at ZT3-10 with an increase at ZT 16, 17, 20 23, and 24 (H). 3.0 mg/kg donepezil decreased duration of REM sleep at ZT 4-6 and increased REM sleep duration at ZT 9 and 20, while 10 mg/kg donepezil decreased REM sleep duration at ZT 4-11, and increased REM sleep duration at ZT 16, 17, 20, 21, 23 and 24. (I). Grey shading represents 12-hour dark period. Data are means ± S.E.M of 1-hour bins; n=8-12/group; open symbols, $p<0.05$ compared to vehicle (Dunnett's test).

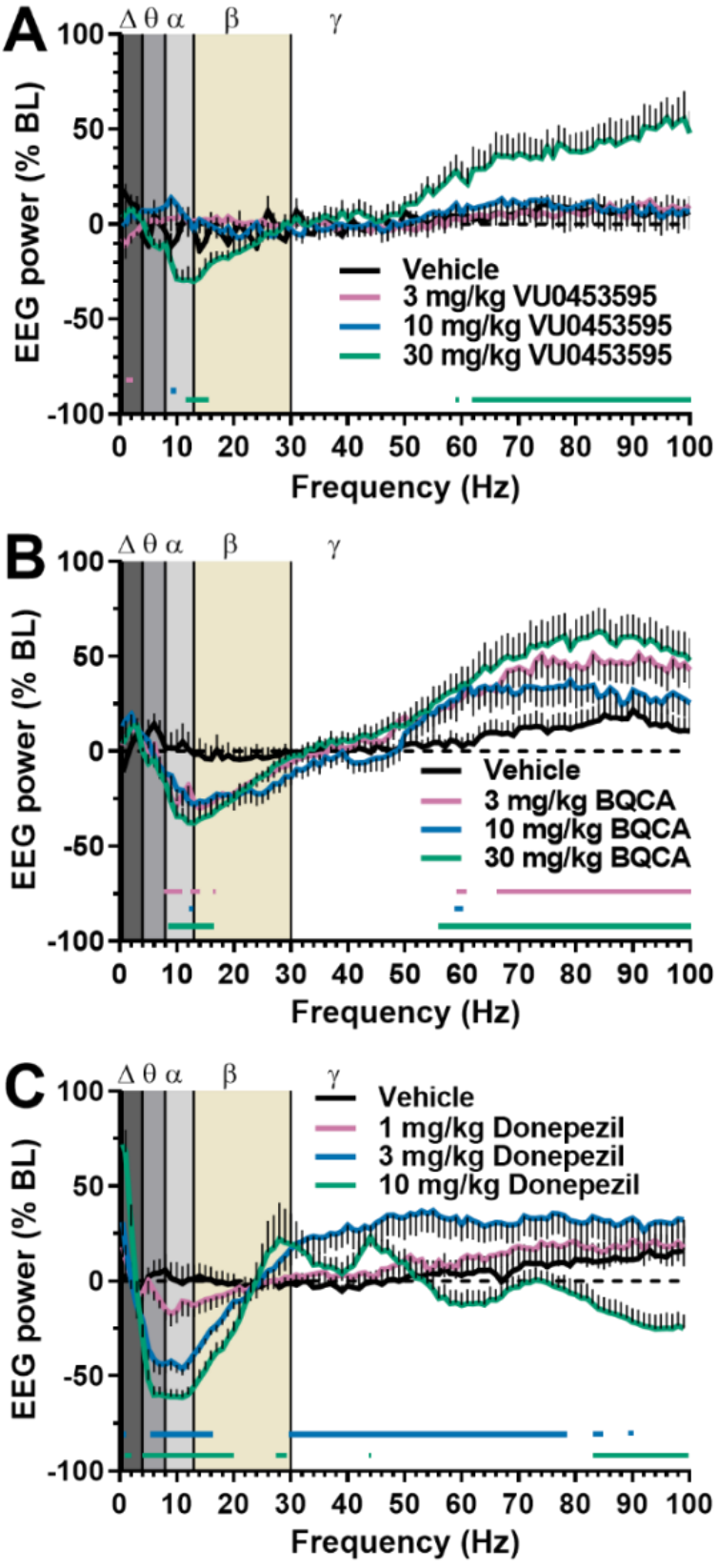
A
Δ θ α
β
γ
EEG power (% BL)
100
50
0
-50
-100
0 10 20 30 40 50 60 70 80 90 100
Frequency (Hz)
Vehicle
3 mg/kg VU0453595
10 mg/kg VU0453595
30 mg/kg VU0453595
B
Δ θ α
β
γ
EEG power (% BL)
Frequency (Hz)
Vehicle
3 mg/kg BQCA
10 mg/kg BQCA
30 mg/kg BQCA
C
Δ θ α
β
γ
EEG power (% BL)
Frequency (Hz)
Vehicle
1 mg/kg Donepezil
3 mg/kg Donepezil
10 mg/kg Donepezil

Figure 3.2. M_1 mAChR PAMs VU0453595 and BQCA, but not donepezil increased high frequency gamma power during wake in young adult rats. Shown are changes in relative spectral power in the frontal cortex, during waking epochs only, in the 1- to 2-hour period following administration of VU0453595 (A), BQCA (B), and Donepezil (C). 3 mg /kg VU0453595 decreased frequencies 0.5 and 1. 10 mg/kg VU0453595 increased frequency 10. 30 mg/kg VU0453595 decreased frequencies 11-13 and increased frequencies 60, and 62-99 (A). 3 mg/kg BQCA decreased frequencies 9-11, 13, 14, 17 and increased frequencies 60-62, and 67-99. 10 mg/kg BQCA decreased frequencies 12, and 13 and increased frequencies 60, and 61. 30 mg/kg BQCA decreased frequencies 9-17 and increased frequencies 57-99 (B). 3 mg/kg donepezil decreased frequencies 0.5, 5-17 and increased frequencies 31-79, 83-85 and 90. 10 mg/kg donepezil decreased frequencies 0.5-2, 4-20, 83-99 and increased frequencies 28, 29, 44 (C). Gray/tan shading represents frequency bands (∆, 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). Data are means ± S.E.M.; n=7-12/group; corresponding-colored horizontal dots/lines represent frequencies at which each dose group was statistically different from vehicle-treated rats, p<0.05, Dunnett's *post hoc* test.

8Hz), Alpha (9-13Hz), Beta (14-30Hz), Low Gamma (31-50Hz). For NHP studies, spectral power for the respective vehicle-treatment for each compound (VU0453595, xanomeline and donepezil) and following dosing with that compound were expressed as a percent of the 3-day vehicle mean.

3.3. Results

M_1 mAChR PAM VU0453595, BQCA, and donepezil produced differential effects on sleep/wake architecture in young rat when dosed 2 hr into the inactive period.

The M_1 mAChR PAM VU0453595 did not alter sleep/wake architecture in young adult rats. There was a main effect of time for all three stages (Wake, NREM, REM; all p<0.001; and a main effect of VU0453595 dose for REM sleep only (p<0.05), but no interaction for any stage (Figure 3.1A-C; see Table 3.2 for details). There was a main effect of time and time by dose interaction for BQCA on time awake and NREM sleep (Figures 3.1D,E; all p<0.001). There was a main effect of time (p<0.001) on REM sleep duration (Figure 3.1F). BQCA increased time awake and decreased NREM and REM sleep.

In contrast, there was a main effect of donepezil dose, time, and dose x time interaction on duration of time awake (Figure 3.1G; all p<0.001), NREM sleep (Figure 3.1H; p<0.001), and REM sleep time (effect of dose (p<0.05), time, and dose x time interaction (both p<0.001) (Figure 3.1I). Donepezil increased time awake and decreased NREM and REM sleep.

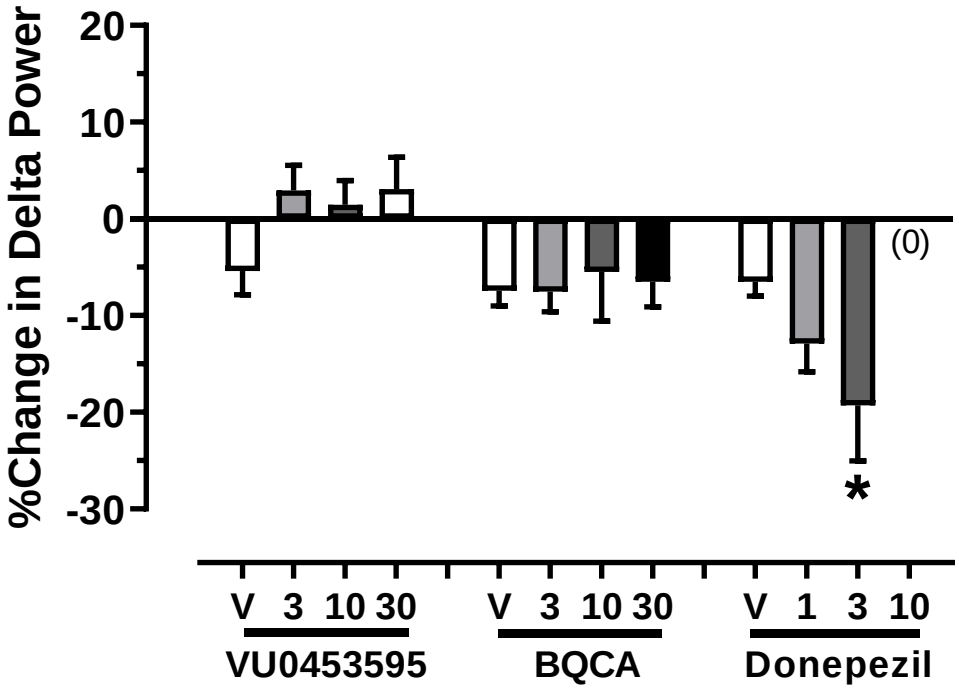

Figure 3.3. Donepezil but not M_1 mAChR PAMs, decrease slow wave sleep quality in rats. Percent change in delta power (0.5-4 Hz) from time periods 1-2 hours following administration compared to the 1-hr baseline period prior to compound administration. One-way ANOVA's conducted for each compound (Donepezil, [$F_{2,22}$=8.38; p<0.01], BQCA, [$F_{3,40}$=0.96; p>0.05], VU0453595, [$F_{3,33}$=2.40; p>0.05]); * p<0.05 compared to respective vehicle (Dunnett's test). 10 mg/kg Donepezil induced wakefulness for the duration of the hour; no sleep epochs could be evaluated (n=10-12/ dose group, except where noted in parenthesis).

M1 mAChR PAM VU0453595, BQCA, and donepezil produced differential effects on relative spectral power in awake epochs 1-2 hrs post dosing.

There was a main effect of frequency and dose x frequency interaction (both p<0.001) of VU0453595 on spectral power. 3.0 mg/kg VU0453595 decreased power in the delta band (red horizontal line), while 30 mg/kg VU0453595 (green horizontal line) decreased power in alpha band and increased power in the high gamma band in the frontal cortex (Figure 3.2A). There was a main effect of frequency and dose x frequency interaction (both p<0.001) of BQCA on spectral power. BQCA decreased power in alpha and low-beta ranges and increased power in the gamma band in the frontal cortex (Figure 3.2B). There was a main effect of dose (p=0.001), frequency and dose x frequency interaction (both p<0.001) of donepezil on spectral power (Figure 3.2C).

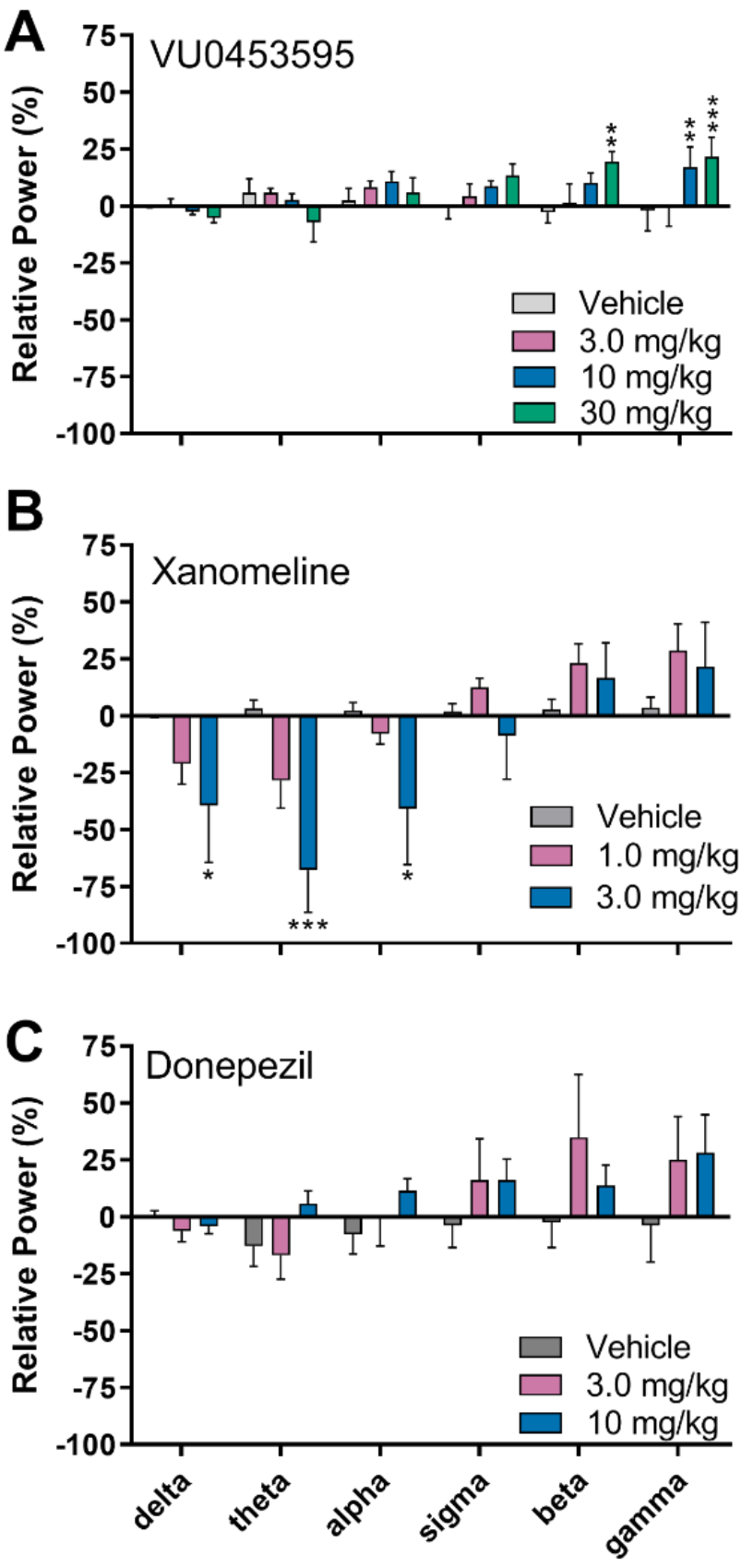
A
VU0453595
Relative Power (%)
75
50
25
0
-25
-50
-75
-100
Vehicle
3.0 mg/kg
10 mg/kg
30 mg/kg
B
Xanomeline
Relative Power (%)
Vehicle
1.0 mg/kg
3.0 mg/kg
C
Donepezil
Relative Power (%)
Vehicle
3.0 mg/kg
10 mg/kg
delta
theta
alpha
sigma
beta
gamma

Figure 3.4. M_1 mAChR PAM VU0453595 increased high frequency gamma power in young adult male cynomolgus macaques. Shown are changes in relative spectral power collapsed into spectral bands (to minimize variability) following administration of VU0453595 (A), xanomeline (B), and donepezil (C) immediately after light onset; power bands are defined as delta (0.5-4Hz), theta (4-8 Hz), alpha (8-13 Hz), sigma (13-18 Hz), beta (18-30 Hz), gamma (30-50 Hz). All 10-second epochs during the first 4 hours post dosing were combined and expressed as a percent change from spectral power within the same frequency band and time period from a mean of 3 vehicle-treated conditions. Data are presented as mean ± S.E.M.; n=5 (VU0453595), n=4 (xanomeline, donepezil); * $p<0.05$ compared to vehicle (Dunnett's test).

Specifically, 3.0 and 10 mg/kg donepezil (blue and green lines respectively) increased low frequency delta power, and decreased power in theta, alpha and low beta ranges. 3.0 mg/kg donepezil increased, whereas 10 mg/kg donepezil decreased power in the gamma band range.

In NREM sleep epochs, 1-2 hrs following dosing, donepezil decreased delta power ($p<0.01$; a measure of sleep quality) whereas BQCA and VU0453595 did not significantly impact delta power ($p>0.05$; see Figure 3.3).

M_1 mAChR PAM VU0453595 increased high frequency beta and gamma power in young adult male NHPs.

qEEG effects seen in rodents translate to young cynomolgus NHPs when dosed 30 minutes after lights are turned on. Neither VU0453595, xanomeline, nor donepezil altered the duration of time in wake, NREM sleep, and REM sleep states (since dosing occurred during early active period, long lasting effects on sleep were not expected; hence, data not shown). For VU0453595, qEEG analysis revealed a dose x frequency interaction ($p<0.01$; Figure 3.4A). 10 and 30 mg/kg increased power in the gamma band frequency and 30 mg/kg increased power in beta power. Xanomeline treatment caused a significant main effect of band ($p<0.01$) and dose x frequency interaction ($p<0.05$; Figure 3.4B), with 3.0 mg/kg xanomeline decreasing power in the delta, theta and alpha bands. There was no significant main effect of donepezil on frequency band, dose nor a dose x frequency interaction (all $p>0.05$), despite qualitative increases in beta (3.0 mg/kg) and gamma bands (3.0 and 10 mg/kg) (Figure 3.4C).

M_1 mAChR PAM VU0453595 displayed a reduced adverse side effect profile compared to xanomeline and donepezil in young adult male NHPs.

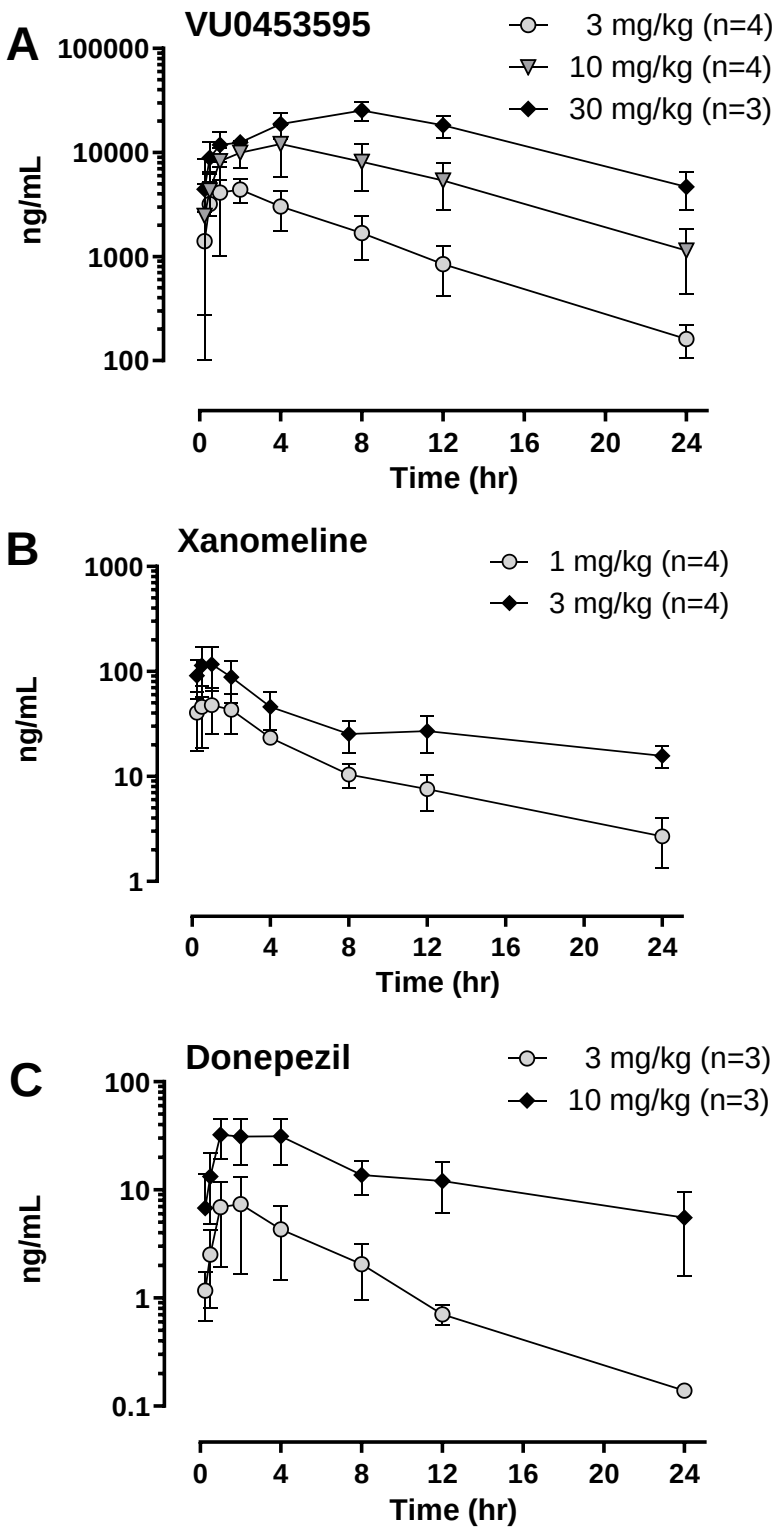

Figure 3.5. Plasma concentration time curves following administration of VU0453595, xanomeline, and donepezil in cynomolgus macaques. Data are means ± S.D, n =3-4/group.

All three compounds demonstrated dose-proportional increases in plasma concentrations; VU0453595 demonstrated a relatively long rate of elimination following oral administration (Figure 3.5). VU0454595 (3.0-30 mg/kg) appeared to cause a slight increase in urination, and a qualitatively assessed decrease in respiration rate at the 30 mg/kg dose (Table 3.3). Occasional changes in posture and motor coordination and leg weakness was noted in one NHP following 30 mg/kg VU0453595 (Table 3.3). To confirm sensitivity of this scale, donepezil and xanomeline were also examined. Xanomeline (1.0 and 3.0 mg/kg) induced miosis, vasocontriction, increased arousal, irritability, salivation and in some NHPs 3.0 mg/kg induced oral dyskinesias (Table 3.4). Donepezil (10 mg/kg) induced urination, defecation, emesis, ptosis, vasoconstriction, irritability, and in some cases tremors (Table 3.5).

M_1 mAChR PAM VU0453595 attenuated reductions in REM sleep in aged wildtype mice when dosed 2 hr into the inactive period.

In aged wildtype mice, there was a significant effect of time ($p<0.001$; Figure 3.6A) and dose x time interaction, ($p<0.01$), on duration of time awake in 2-h bins across the 24-h period. There was a significant effect of time ($p<0.001$; Figure 3.6B) and dose x time interaction ($p<0.05$), on duration of NREM sleep time. There was a significant effect of time and dose x time interaction (both $p<0.001$; Figure 3.6C), on duration of REM sleep.

In young wildtype mice, there was a significant effect of time ($p<0.001$; Figure 3.6D) and VU0453595 dose ($p<0.05$) on duration of time awake in 2 h bins across the 24-h period. There was a significant effect of time ($p<0.001$; Figure 3.6E) and dose x time interaction ($p<0.05$) on duration of NREM sleep time. In contrast, there was a significant effect of time ($p<0.001$; Figure 3.6F) on duration of REM sleep time.

VU0453595 transiently increased wake and decreased NREM sleep followed by sustained increases in REM sleep duration (Figure 3.6A-C) in aged wildtype mice. Similar transient effects were present on wake and NREM sleep but no effects on REM sleep were present in young wildtype mice (Figures 3.6D-F).

Species	Experiment	Measure	Statistical Test	Comparison	Degrees of freedom	F	p	*	Group size	Figure	Post hoc results (Dunnett's test unless noted otherwise)
Rat	VU0453595 on time awake	Duration (min/hr)	Repeated Measures Mixed-Effects Model	Dose	3, 33	0.137	0.937		8-9	1A	None
				Time	23, 253	31.030	<0.001	***			
				Dose x Time	69, 423	1.002	0.479				
Rat	VU0453595 on time in NREM	Duration (min/hr)	Repeated Measures Mixed-Effects Model	Dose	3, 33	0.291	0.831		8-9	1B	None
				Time	23, 253	34.400	<0.001	***			
				Dose x Time	69, 423	1.076	0.328				
Rat	VU0453595 on time in REM	Duration (min/hr)	Repeated Measures Mixed-Effects Model	Dose	3, 33	3.636	0.023	*	8-9	1C	None
				Time	23, 253	14.450	<0.001	***			
				Dose x Time	69, 423	1.294	0.068				
Rat	BQCA on time awake	Duration (min/hr)	Repeated Measures Two-Way ANOVA	Dose	3, 33	2.494	0.077		12	1D	3 mg/kg vs Veh: ZT 13 10 mg/kg vs Veh: ZT 4,6 30 mg/kg vs Veh: ZT 4,5,13,14,22
				Time	23, 253	56.520	<0.001	***			
				Dose x Time	69,759	1.928	<0.001	***			
Rat	BQCA on time in NREM	Duration (min/hr)	Repeated Measures Two-Way ANOVA	Dose	3, 33	1.687	0.189		12	1E	10 mg/kg vs Veh: ZT 4,6 30 mg/kg vs Veh: ZT 4-6,13-14,22
				Time	23, 253	56.020	<0.001	***			
				Dose x Time	69, 759	1.798	<0.001	***			
Rat	BQCA on time in REM	Duration (min/hr)	Repeated Measures Two-Way ANOVA	Dose	3, 33	1.267	0.302		12	1F	30 mg/kg vs Veh: ZT 4,13-14
				Time	23, 253	21.530	<0.001	***			
				Dose x Time	69, 759	1.258	1.258				
Rat	Donepezil on time awake	Duration (min/hr)	Repeated Measures Two-Way ANOVA	Dose	3, 27	11.140	<0.001	***	10	1G	1.0 mg/kg vs Veh: ZT 3-4, 24 3.0 mg/kg vs Veh: ZT 3-7,16, 20,23-24 10 mg/kg vs Veh: ZT 3-11,16-17, 20, 23-24
				Time	23, 207	24.030	<0.001	***			
				Dose x Time	69, 621	12.310	<0.001	***			
Rat	Donepezil on time in NREM	Duration (min/hr)	Repeated Measures Two-Way ANOVA	Dose	3, 27	9.681	<0.001	***	10	1H	1.0 mg/kg vs Veh: ZT 3-4,24 3.0 mg/kg vs Veh: ZT 3-7,16,20,23 10 mg/kg vs Veh: ZT 3-10,16,17,20, 23-24
				Time	23, 207	23.270	<0.001	***			
				Dose x Time	69, 621	12.070	<0.001	***			
Rat	Donepezil on time in REM	Duration (min/hr)	Repeated Measures Two-Way ANOVA	Dose	3, 27	4.445	0.012	*	10	1I	3.0 mg/kg vs Veh: ZT 4-6,9,20 10 mg/kg vs Veh: ZT 4-11, 16-17, 20-21,23-24
				Time	23, 207	8.201	<0.001	***			
				Dose x Time	69, 621	5.464	<0.001	***			
Rat	VU0453595 on qEEG	% Change from BL	Repeated Measures Mixed-Effects Model	Dose	3, 27	2.731	0.063		7-9	2A	3 mg/kg vs Veh: Freq 0.5,1 10 mg/kg vs Veh: Freq 9 30 mg/kg vs Veh: Freq 11-13, 60,62-99
				Frequency	100, 900	9.606	<0.001	***			
				Dose x Frequency	300, 1791	3.569	<0.001	***			
Rat	BQCA on qEEG	% Change from BL	Repeated Measures Mixed-Effects Model	Dose	3, 33	2.602	0.069		10-12	2B	3 mg/kg vs Veh: Freq 9-11,13-14,17,60-62,67-99 10 mg/kg vs Veh: Freq 12-13,60-61 30 mg/kg vs Veh: Freq 9-17,57-99
				Frequency	100, 1100	41.400	<0.001	***			
				Dose x Frequency	300, 3098	3.295	<0.001	***			
Rat	Donepezil on qEEG	% Change from BL	Repeated Measures Two-Way ANOVA	Dose	3, 27	7.217	0.001	**	10	2C	3 mg/kg vs Veh: Freq 0.5, 5-17,31-79,83-85,90 10 mg/kg vs Veh: Freq 0.5-2,4-20,28,29,44,83-99
				Frequency	99, 891	16.830	<0.001	***			
				Dose x Frequency	297, 2673	5.611	<0.001	***			

Species	Experiment	Measure	Statistical Test	Comparison	Degrees of freedom	F	p	*	Group size	Figure	Post hoc results (Dunnett's test unless noted otherwise)
NHP	VU0453595 on qEEG	% Change from BL	Repeated Measures Two-Way ANOVA	Dose	3, 12	0.930	0.456		5	3A	10 mg/kg vs Veh: Low Gamma 30 mg/kg vs Veh: Beta and Low Gamma
				Frequency Band	5, 20	2.706	0.050				
				Dose x Frequency	15, 60	2.720	0.003	**			
NHP	Xanomeline on qEEG	% Change from BL	Repeated Measures Two-Way ANOVA	Dose	2, 6	3.911	0.082		4	3B	3.0 mg/kg vs Veh: Delta, Theta, Alpha
				Frequency Band	5, 15	6.189	< 0.01	**			
				Dose x Frequency	10, 30	2.305	<0.05	*			
NHP	Donepezil on qEEG	% Change from BL	Repeated Measures Two-Way ANOVA	Dose	2, 6	1.396	>0.05		4	3C	NA
				Frequency Band	5, 15	2.715	>0.05				
				Dose x Frequency	10, 30	1.650	>0.05				
Aged Mice	VU0453595 on time awake	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Dose	3, 27	0.107	>0.05		10	4A	30 mg/kg vs Veh: ZT 4
				Time	11, 99	33.730	<0.001	***			
				Dose x Time	33, 297	1.754	<0.01	**			
Aged Mice	VU0453595 on time in NREM	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Dose	3, 27	1.199	>0.05		10	4B	30 mg/kg vs Veh: ZT 4,6
				Time	11, 99	24.190	<0.001	***			
				Dose x Time	33, 297	1.543	<0.05	*			
Aged Mice	VU0453595 on time in REM	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Dose	3, 27	0.578	>0.05		10	4C	3 mg/kg vs Veh: ZT 6,8,20 10 mg/kg vs Veh: ZT 6,8 30 mg/kg vs Veh: ZT 8,10
				Time	11, 99	21.800	<0.001	***			
				Dose x Time	33, 297	2.201	<0.001	***			
Young Mice	VU0453595 on time awake	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Dose	3, 36	3.236	<0.05	*	13	4D	30 mg/kg vs Veh: ZT 4
				Time	11, 132	74.020	<0.001	***			
				Dose x Time	33, 396	1.239	>0.05				
Young Mice	VU0453595 on time in NREM	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Dose	3, 36	2.404	>0.05		13	4E	10 mg/kg vs Veh: ZT 6, 22 30 mg/kg vs Veh: ZT 4
				Time	11, 132	42.420	<0.001	***			
				Dose x Time	33, 396	1.534	<0.05	*			
Young Mice	VU0453595 on time in REM	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Dose	3, 36	0.418	>0.05		13	4F	NONE
				Time	11, 132	73.030	<0.001	***			
				Dose x Time	33, 396	0.983	>0.05				
Mice	Young vs Aged time awake	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Age	1, 21	0.301	>0.05		10 aged 13 young	4G	(Bonferroni's test) Young vs Aged: ZT 8, 22
				Time	11, 231	30.800	<0.001	***			
				Age x Time	11, 231	3.945	<0.001	***			
Mice	Young vs Aged time in NREM	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Age	1, 21	0.038	>0.05		10 aged 13 young	4H	(Bonferroni's test) NONE
				Time	11, 231	25.690	<0.001	***			
				Age x Time	11, 231	2.664	<0.01	**			
Mice	Young vs Aged time in REM	Duration (min/2hrs)	Repeated Measures Two-Way ANOVA	Age	1, 21	24.820	<0.001	***	10 aged 13 young	4I	(Bonferroni's test) Young vs Aged: ZT 6-10, 22
				Time	11, 231	21.140	<0.001	***			
				Age x Time	11, 231	9.310	<0.001	***			
Young Mice	VU0453595 on qEEG	% Change from BL	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 33	9.513	0.001	***	11-12	5A	30 mg/kg vs Veh: Freq 0.5-2, 30-50
				Frequency	50, 550	2.647	<0.001	***			
				Dose x Frequency	150, 1497	7.070	<0.001	***			
Aged Mice	VU0453595 on qEEG	% Change from BL	Repeated Measures Two-Way ANOVA	Dose	3, 30	2.275	0.100		10-11	5B	10 mg/kg vs Veh: Freq 3,40,43-50 30 mg/kg vs Veh: Freq 0.5-2,5-6,44,46-50
				Frequency	50, 500	4.042	<0.001	***			
				Dose x Frequency	150, 1347	3.263	<0.001	***			
Mice	Young vs Aged on qEEG	% Change from BL	Repeated Measures Two-Way ANOVA	Age	1, 20	0.289	0.597		10-12	5C	(Bonferroni's test) Young vs Aged: Freq 0.5, 3
				Frequency	50, 1000	2.555	<0.001	***			
				Dose x Frequency	50, 1000	2.555	<0.001	***			

Table 3.2. Full statistical analysis

VU0453595 (i.g.)				3 mg/kg (n=4)							10 mg/kg (n=4)										30 mg/kg (n=4)									
Time Point	0	0.25	0.5	1	2	4	8	12	24		0	0.25	0.5	1	2	4	8	12	24		0	0.25	0.5	1	2	4	8	12	24
Autonomic Nervous System																														
Salivation	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Urination	-	-	+	-	+	-	+	++	+		-	+	-	-	+	+	+	-	-		-	-	-	-	+	-	-	-	-
Defecation (amt)	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	+	+	-	-	-	-	-
Defecation (consistency)	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Emesis	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Miosis	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Mydriasis	-	+	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	+	-	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Exophtalmus	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Respiratory rate	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	++↓	+↓	+↓	-	-	-	-	-
Penile erection	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	+	+	+	-	-	-	-
Yawn	-	-	-	-	+	-	-	-	-		-	-	-	-	+	-	-	-	-		-	-	-	-	-	-	-	-	-
Vasodilatation	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Vasoconstriction	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Irritability	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	+	-	-	-	-	-	-
Somatomotor System																														
Physical Appearance	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Tremors	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Catalepsy	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Leg weakness	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	+	+	-	-	-	-	-	-
VisuoMotor Coordination	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	+	+	-	-	-	-	-	-
Posture	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Unrest	-	-	-	-	-	-	-	-	-		-	-	-	+	-	-	-	-	-		-	-	-	+	-	+	-	-	-
Stereotypies	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	+	-	-	-	-	-	-
Arousal	-	-	-	-	-	-	-	-	-		-	+	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Sedation	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Oral Dyskinesia	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Bradykinesia	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Dystonia	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Grooming	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Vocalization	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Self-injurious Behavior	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-

The effects of VU0453595 (3.0, 10.0, 30.0 mg/kg IG) on autonomic and somatomotor system function were evaluated. The mean scores of 3 monkeys are classified as follows; -,= no effect; +, 0.01-0.25; ++ 0.26-0.50; +++, 0.51-0.75; ++++, 0.76-1.00

Table 3.3. Adverse effect profiling of VU0453595 in cynomolgus macaques

Xanomeline (s.c.)	1.0 mg/kg (n=4)									3.0 mg/kg (n=4)								
Time Point	0	0.25	0.5	1	2	4	8	12	24	0	0.25	0.5	1	2	4	8	12	24
Autonomic Nervous System																		
Salivation	-	-	+	++	++	-	-	-	-	-	+	++++	+++	+	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urination	-	-	-	-	-	++	+	-	-	-	-	-	-	+	-	+	+	-
Defecation (amount)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Defecation (consistency)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Emesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Miosis	-	-	-	+	+++	+++	+	-	-	-	-	+	^	^^^	^^	++	+	-
Mydriasis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ptosis	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Exophtalmus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiratory rate	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Penile erection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yawn	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Vasodilatation	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vasoconstriction	-	-	-	-	+	+	+	-	-	-	++	++	++	+	-	-	-	-
Irritability	-	-	++	+	+	+	+	-	-	-	-	++	++++	+++	++	+	-	-
Somatomotor Systems																		
Physical Appearance	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Tremors	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalepsy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leg weakness	-	-	-	-	-	-	-	-	-	-	++	+	-	-	-	-	-	-
VisuoMotor Coordination	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Posture	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Unrest	-	-	+	++++	++++	++	++	-	-	-	++	+++	++++	++++	++	+	+	-
Stereotypies	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arousal	-	-	++	+++	+	+	-	-	-	-	+	++	++	++++	++	+	-	-
Sedation	-	-	-	+	+	+	-	-	-	-	+	-	++	+	-	-	-	-
Oral Dyskinesia	-	-	-	-	-	-	-	-	-	-	++	+	+	-	-	-	-	-
Bradykinesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dystonia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grooming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vocalization	-	-	++	-	++	+	+	+	-	-	+	+	+	++	+	-	-	-
*Self-injurious Behavior**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The effects of Xanomeline (1.0, 3.0 mg/kg SC) on autonomic and somatomotor system function were evaluated. The mean scores of 3 monkeys are classified as follows; -,=no effect; +, 0.01-0.25; ++ 0.26-0.50; +++, 0.51-0.75; ++++, 0.76-1.00; ^, 1.01-1.25; ^^1.26-1.50; ^^^,

Table 3.4. Adverse effect profiling of Xanomeline in cynomolgus macaques

Donepezil (i.g.)	3 mg/kg (n=3)									5.6 mg/kg (n=2)									10 mg/kg (n=3)								
Autonomic Nervous System																											
Time Point	0	0.25	0.5	1	2	4	8	12	24	0	0.25	0.5	1	2	4	8	12	24	0	0.25	0.5	1	2	4	8	12	24
Salivation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urination	-	-	-	-	-	++	-	-	-	-	-	-	-	++	-	-	-	++	-	-	-	++	++	++	+++	++	-
Defecation (amt)	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++	+++	++	-	-	-
Defecation (consistency 1 soft 2 diarrhea)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+++	++	-	-	-
Emesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
Miosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mydriasis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++	++++	-	-	-
Exophtalmus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiratory rate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penile erection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yawn	-	-	+++	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vasodilatation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vasoconstriction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	++++	+++	-	-	-
Irritability temp. (°C)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-
Somatomotor Systems																											
Physical Appearance	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremors	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+++	+++	-	-	-
Catalepsy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leg weakness	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VisuoMotor Coordination	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Posture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unrest	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-
Stereotypies	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arousal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sedation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-
Oral Dyskinesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-
Bradykinesia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dystonia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grooming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vocalization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-
*Self-injurious Behavior**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The effects of donepezil (3, 5.6, 10 mg/kg IG) on autonomic and somatomotor system function were evaluated. The mean scores of 3 monkeys are classified as follows; -,=no effect; +, 0.01-0.25; ++ 0.26-0.50; +++, 0.51-0.75; ++++, 0.76-1.00

Table 3.5. Adverse effect profiling of donepezil in cynomolgus macaques

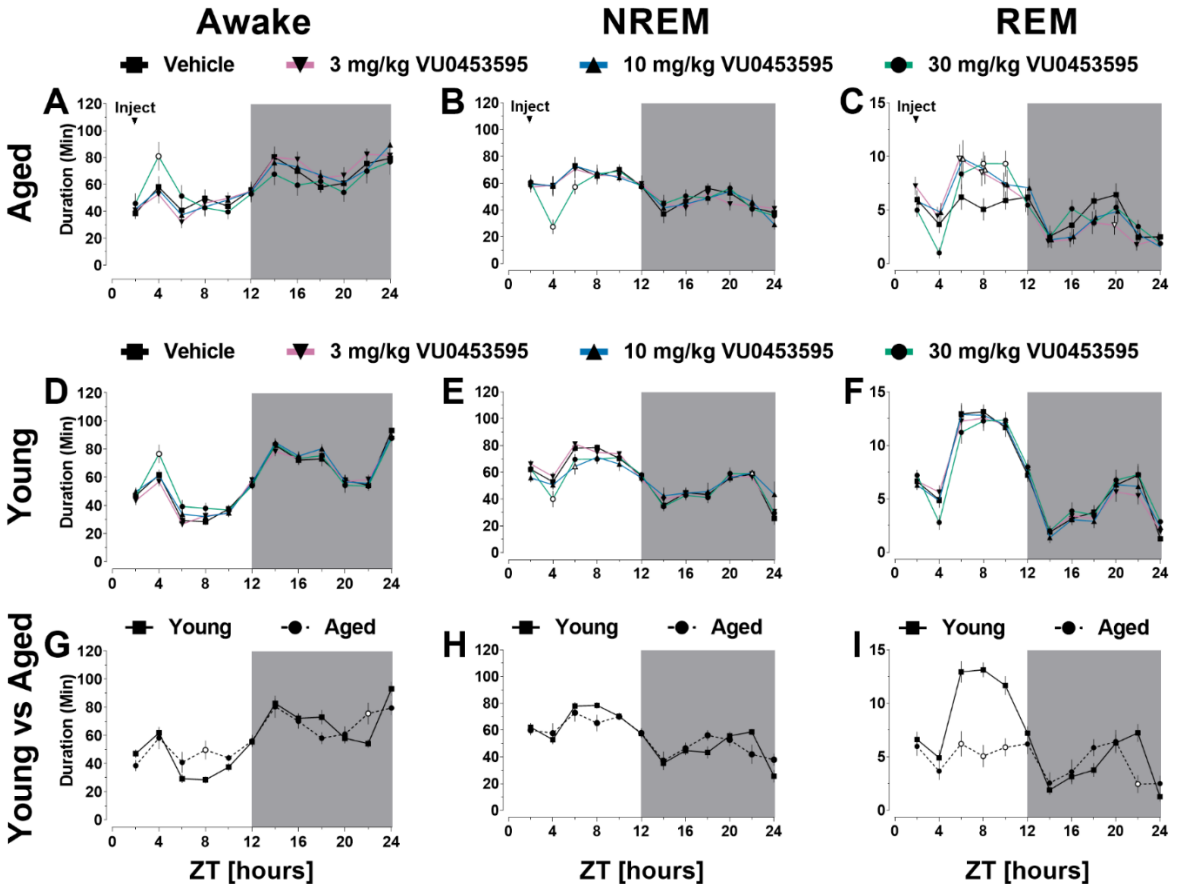

Figure 3.6. M_1 mAChR PAM VU0453595 attenuated age-related deficits in REM sleep in aged wildtype mice. Shown is the duration of time awake (A, D, G), in non-REM (NREM) sleep (B, E, H), or in REM sleep (C, F, I) in aged (22-26 month-old [A-C]) and young adult (4-6 month-old) mice (D-F). Following compound administration 2 hours into the light (quiescent) phase (see arrowhead), VU453595 increased REM sleep duration in aged (22-26 month-old) mice (C) without affecting REM sleep in young (4-6 month-old) mice (F). Specifically, 30 mg/kg VU0453595 produced a significant increase in duration of time awake at ZT 4 (A) and a decrease in NREM sleep from ZT4 and 6 in aged mice (B). 3 mg/kg VU0453595 increased REM sleep from across ZT 6, 8 and 20, 10 mg/kg VU0453595 increased REM sleep at ZT 6 and 8 and 30 mg/kg VU0453595 increased REM sleep duration from ZT 8 and 10 in aged wildtype mice (C). In young wildtype mice, 30 mg/kg VU0453595 produced significant increases in duration of time awake at ZT 4 (D). 10 mg/kg VU0453595 produced a significant decrease in duration of NREM sleep at ZT 6 and 22 and 30 mg/kg VU0453595 produced a significant decrease in NREM sleep from ZT 4 (E); but no significant effects on REM sleep duration (F). For comparison, vehicle-treated groups were replotted in G-I to better illustrate the age-related decreases in REM sleep duration. Aged mice showed significantly higher durations of time awake at ZT 8 and 22 (G) and significantly lower durations of REM sleep at the ZT 6-10 and 22 time points compared to young mice (I).Grey shading represents 12-hour dark period. Data are means ± S.E.M. of 2-hour bins; n=10-13/group; Open symbols, $p<0.05$ compared to vehicle (Dunnett's test A-F; Bonferroni G-I).

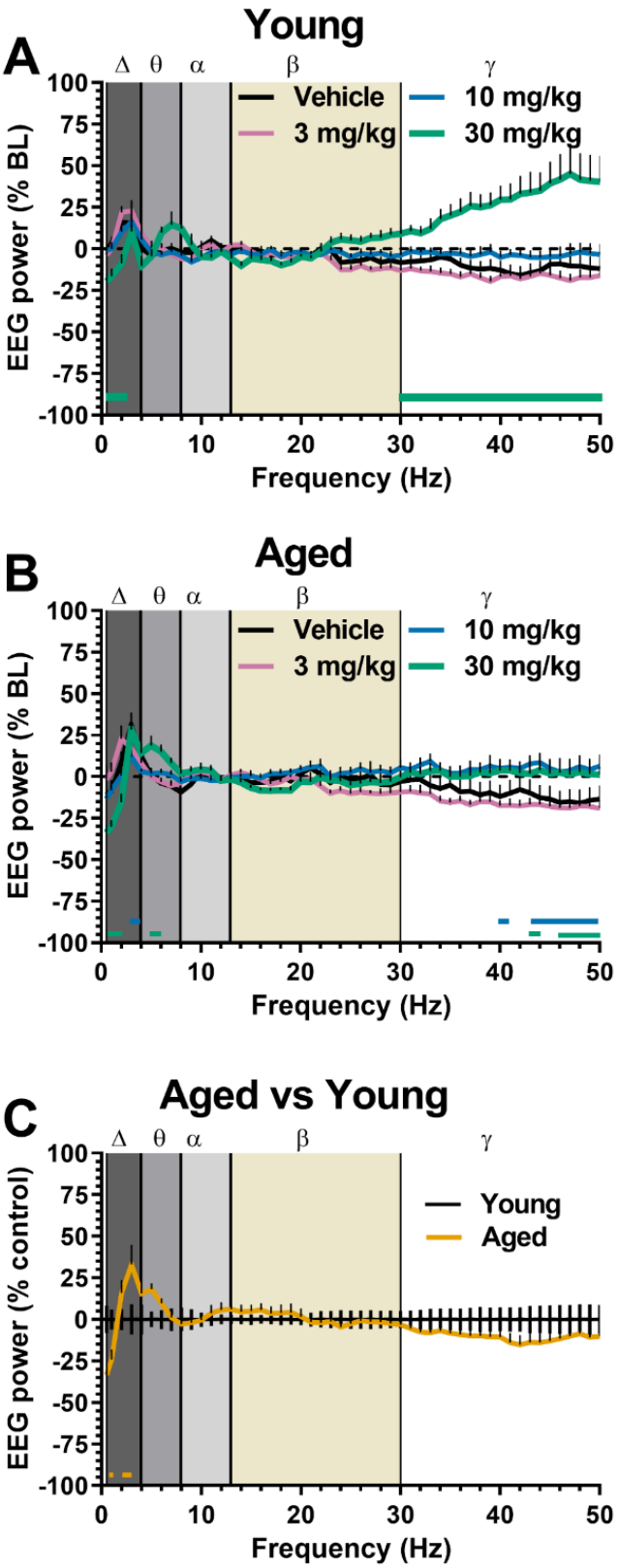
A
Young
Δ θ α β γ
Vehicle
3 mg/kg
10 mg/kg
30 mg/kg
EEG power (% BL)
Frequency (Hz)
B
Aged
Δ θ α β γ
Vehicle
3 mg/kg
10 mg/kg
30 mg/kg
EEG power (% BL)
Frequency (Hz)
C
Aged vs Young
Δ θ α β γ
Young
Aged
EEG power (% control)
Frequency (Hz)

Figure 3.7. M_1 mAChR PAM VU0453595 produced changes in high frequency gamma power in young and aged wildtype mice. Shown are changes in relative spectral power in the frontal cortex, during waking epochs only, in the 1- to 2-hour period following administration of VU0453595 in young adult (4-6 month-old [A]) and aged (22-26 month-old) wildtype mice (B). In young wildtype mice, 30 mg/kg VU0453595 decreased power distribution in 0.5-2 range and increased power at 30-50 Hz (A). In aged wildtype mice, 10 mg/kg VU0453595 decreased power distribution at 3 HZ, and increased power at 40, and 43-50 Hz; 30 mg/kg VU0453595 decreased power distribution at 0.5-2 Hz and increased power at 5-6, 44, and 46-50 HZ (B). Comparison of spectral power in vehicle-treated young and aged mice shown as percent difference from young mice (C). Aged mice showed a decrease at 0.5 Hz and an increase at 3 Hz compared to young wildtype mice. Gray/tan shading represents 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). Data are means ± S.E.M.; in (C), error bars on the young mice group represent SEM following calculations of individual percent differences from the group mean. n=11-12/group; corresponding-colored horizontal dots/lines represent frequencies at each dose that were statistically different from vehicle-treated rats. $p<0.05$ (Dunnett's *post hoc* test).

When comparing vehicle-treated aged wildtype mice to vehicle-treated young wildtype mice there was a significant effect of time (Figure 3.6G) and time x age interaction (both $p<0.001$) on duration of time awake in 2 h bins across the 24-h period. There was a significant effect of time ($p<0.001$; Figure 3.6H) and time x age interaction ($p<0.01$) on duration of NREM sleep in 2 h bins across the 24-h period. There was a significant effect of age (Figure 3.6I), time and time x age interaction (all $p<0.001$) on duration of REM sleep in 2 h bins across the 24-h period. Aged wildtype mice showed greater time awake and less time in REM sleep compared to young mice (Figure 3.6G, I).

M_1 mAChR PAM VU0453595 increased high frequency gamma power in awake epochs 1-2 hrs post dosing in young and aged mice.

In young wildtype mice there was a main effect of VU453595 dose, frequency, and dose x frequency interaction (all $p<0.001$) on spectral power in the frontal cortex (Figure 3.7A). In aged wildtype mice (Figure 3.7B), there was a significant effect of frequency and dose by frequency interaction (both $p<0.001$). In both young and aged mice, VU0453595 increased high frequency gamma power and 30 mg/kg VU0453595 decreased power in the low frequency range. The effects of 30 mg/kg VU0453595 on sleep-wake architecture and qEEG were absent in M_1 knockout mice (Figure 3.8). Comparison of aged, vehicle-treated mice to young vehicle-treated mice revealed a significant effect of frequency ($p<0.0001$) and frequency x age interaction ($p<0.0001$; Figure 3.7C).

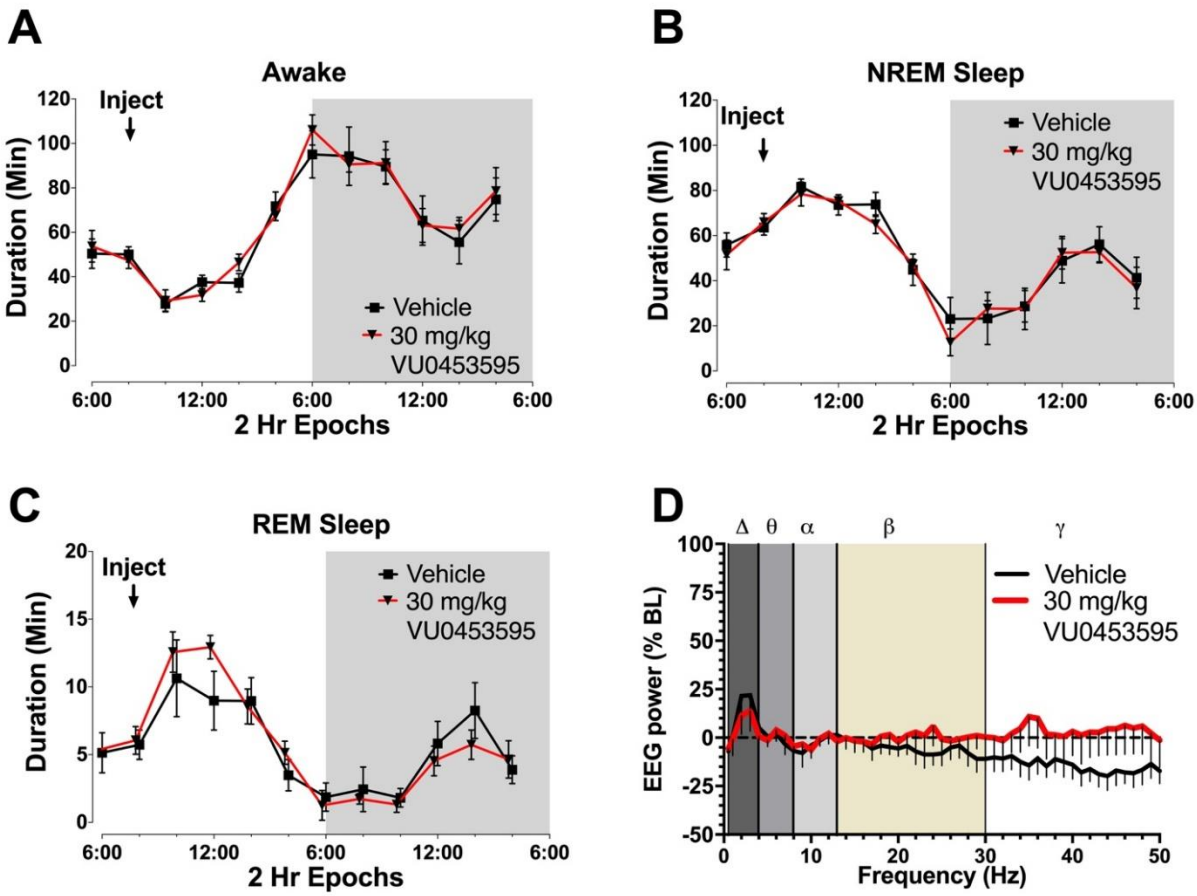

Figure 3.8. Effects of VU0453595 are absent in young M_1 KO mice. To confirm selectivity of the M_1 PAM, we administered vehicle or 30 mg/kg VU0453595 to young M_1 mAChR KO mice and examined sleep/wake architecture and relative spectral power. Changes in duration of time in each state [awake (A), NREM (B), or REM (C) sleep] over 24-hr period following administration of Vehicle or 30 mg/kg VU0453595 2 hrs into the quiescent period. Two-way RM ANOVA followed by Tukey's post-hoc test; grey rectangular represents 12-hour dark period. (D) Changes in relative spectral power in the frontal cortex during waking epochs only, during the 1-2 h period following administration of VU0453595 in young M_1 KO mice. Data from waking epochs only were averaged and expressed as a percent change from awake epochs 1-hr prior to administration across the entire power spectrum (0.5-100 Hz). Relative power is summed in 1 Hz bins (0.5-100 Hz) from all 10-sec waking epochs and expressed as a percent change (± SEM) from respective power within the same frequency bin during waking epochs from the 1 h baseline (BL) period prior to dosing. As shown in panels A-C, there was a main effect of time but not VU0453595 dose nor interaction on duration of time wake in NREM or REM sleep (Awake: time ($[F_{3.6,16.8}=19.85, p<0.0001]$, dose $[F_{1,5}=0.29, p>0.05]$ or dose x time interaction $[F_{2.7,13.9}=0.36, p>0.05]$; NREM: time $[F_{3.6,16.8}=17.54, p<0.0001]$, dose $[F_{1,5}=0.43, p>0.05]$ or dose x time interaction $[F_{2.9,14.3}=0.35, p>0.05]$; REM: $[F_{11.55}=12.91, p<0.0001]$, dose $[F_{1,5}=0.71, p>0.05]$ or dose x time interaction $[F_{11,55}=1.14, p>0.05]$). In the young M_1 mAChR KO mice (D), there no effect of frequency $[F_{1.7,10}=1.24, p<0.05]$, dose $[F_{1,6}=1.34, p>0.05]$ or interaction $[F_{1.79,10.7}=1.46, p>0.05]$ when comparing 30 mg/kg VU0453595 to vehicle administration. N= 6 M_1 mAChR KO mice.

3.4. Discussion

In the present study, side by side comparisons across young mice, rats and NHPs, the M_1 mAChR PAM VU0453595 produced dose-related increases in high frequency gamma power, a

well-characterized correlate of arousal and cognitive enhancement (Buzsáki and Silva, 2012), without changing the duration of time spent in the different sleep/wake stages. Importantly, these effects were absent in M_1 mAChR KO mice and were observed within a dose-range that did not induce cholinergic-mediated adverse effects previously reported with AChEIs, xanomeline, and other M_1-preferring orthosteric agonists. This qEEG signature of selective M1 mAChR engagement was recapitulated by the M_1 ago-PAM BQCA. In contrast, the AChEI donepezil produced a distinct qEEG signature, dose-dependently increased time awake, nonselectively decreased sleep duration in young rats, and decreased delta power in NREM sleep and so decreased sleep quality (Iwata et al., 2000; Jung et al., 2012; Nissen, Nofzinger, et al., 2006; Riemann et al., 1994). In aged mice, VU0453595 produced a robust attenuation of age-related changes in sleep, specifically enhancement of REM sleep duration which is shown to be significantly decreased with c aging. In combination, these findings in young and aged animals produce an important baseline for the future evaluation of M_1 PAM effects on sleep wake/architecture and EEG in preclinical species and clinical populations.

Interestingly, in aged mice, the magnitude of VU0453595-dependent increases in high frequency gamma power were reduced in comparison with effects observed in young mice. Since an M_1 PAM enhances effects of endogenous ACh, these data suggest possible differences in endogenous ACh signaling which can vary with circadian rhythm, age and disease state. Under normal conditions, stimulation of cholinergic projections from midpontine cholinergic nuclei increases the transition from NREM sleep selectively to REM sleep (van Dort et al., 2015). In contrast, stimulation of the cholinergic basal forebrain neurons during NREM promotes the transition to either REM sleep or wakefulness (Han et al., 2014) and are directly responsible for the increased gamma and theta oscillations during waking states (Cape et al., 2000). The basal forebrain cholinergic system degenerates in AD (Whitehouse, Price, Struble, Clark, Coyle, and Delon, 1982), whereas the midpontine projection neurons are spared (Woolf et al., 1989). As similar though less severe cholinergic changes are present in non-pathological aging, M_1 mAChR

PAMs may enhance ACh-mediated functions through intact midpontine projections resulting in increased REM sleep. However, once age-related basal forebrain cholinergic degeneration becomes severe, insufficient endogenous ACh signaling may preclude an M_1 PAM from having the same magnitude of effect on arousal during wakefulness.

In this study, animals were dosed during the inactive period (when cholinergic tone is presumed to be low) to enable maximal possible dynamic range to observe increases in arousal. However, in aged animals with declining integrity of the central cholinergic system, dosing during the active period may be the optimal time to observe enhancements in arousal. Whereas in young animals enhancing arousal during the inactive period is possible, increasing REM sleep above optimal levels may be difficult.

Several promising M_1 mAChR allosteric modulators have progressed into clinical trials as potential treatments for cognitive impairments associated with AD or neuropsychiatric disorders, including a proof-of-concept study examining efficacy of MK-7622 as an adjunct treatment to AChEIs in AD patients (Voss et al., 2018) Unfortunately these programs were halted due to a lack of true subtype-selectivity and off-target adverse side effect liability (Bradley et al., 2018; Nathan et al., 2013; Uslaner et al., 2018) (Merck, ClinicalTrial ID: NCT01852110). Interestingly, while VU0453595 increased power in high frequency ranges in young NHPs similar to young mice and rats, the M_1 PAM MK-7622 dose-dependently decreased power in delta to sigma power bands in young NHPs (Uslaner et al., 2018), similar to our present finding with xanomeline. Differences between MK-7622 effects and VU0453595 in NHPs may be attributed to methodology (e.g. electrode placement, data collection, analytical techniques). An alternative interpretation is that an increase in high frequency gamma power was achieved by dose escalation that is precluded by dose-limiting adverse effects of less selective M_1 PAMs, direct agonists, or indirect agonists (e.g., MK-7622, xanomeline, donepezil). Indeed, MK-7622 displays robust agonist activity at the M_1 mAChR in cell-based assays, as well as seizure activity (Moran et al., 2018) and cholinergic-mediated adverse effects within dose ranges that improve cognition in rodents (Mandai et al.,

2020); all effects not seen with the M_1 PAM VU0453595 (Moran et al., 2018). While quantitatively different from the actions of VU0453595, qualitatively, both compounds shifted power distribution from lower to higher frequency ranges which likely corresponds with modest increases in arousal and behavioral effects. Importantly, prior studies with MK-7622 were promising in that dose ranges predicted from preclinical studies produced reliable changes on qEEG in healthy humans, notably increased power in sigma and beta power bands in humans (Uslaner et al., 2018). Specific to VU0453595, it remains to be seen whether this increase in gamma will correlate with greater efficacy for cognitive enhancement. Recently, the Warren Center for Neuroscience Drug Discovery completed a Phase I study in healthy volunteers with the M_1 PAM VU319 and revealed dose-related changes in both cognitive and EEG measures of central M_1-mediated target engagement (Conley et al., 2019; P. Newhouse et al., 2019); future studies will assess whether this translates to efficacy in clinical populations.

In summary, the present findings suggest selective M_1 PAMs may be beneficial in enhancing not only cognition and/or arousal, but also in normalizing REM sleep deficits observed in pathologic aging and neurodegenerative diseases with minimal adverse effects and support the utility of EEG as a highly translational marker of central M_1 target engagement for future clinical M_1 PAM development.

Chapter from:

Gould RW, Russell JK, Nedelcovych MT, Bubser M, Blobaum AL, Bridges TM, Newhouse PA, Lindsley CW, Conn PJ, Nader MA, Jones CK. Modulation of arousal and sleep/wake architecture by M_1 PAM VU0453595 across young and aged rodents and nonhuman primates. 2020. Neuropsychopharmacology. Dec;45(13):2219-2228.

CHAPTER 4

The M_1 Muscarinic Acetylcholine Receptor Modulator VU0453595 Normalizes Wake and Arousal Deficits Alone and in Combination with the Acetylcholinesterase Inhibitor Donepezil in Non-pathologically Age Mice

4.1. Introduction

Reductions in cholinergic synaptic markers in cortical and limbic regions, indicating reduced central cholinergic structure, have been identified in non-pathologically aged clinical volunteers (Kanel et al., 2022), AD populations (Aghourian et al., 2017; Whitehouse et al., 1982) and in non-pathologically aged preclinical species (Fischer et al., 1992; Parent et al., 2012; Xie et al., 2019). Of the different cholinergic markers, choline acetyltransferase (ChAT) is commonly utilized preclinically in immunohistochemistry (IHC) studies (Fischer et al., 1992; Xie et al., 2019) and serves as a well-validated marker for central cholinergic structure. This declining basal forebrain cholinergic integrity has been associated with a number of the cognitive, sleep/wake architecture, and arousal deficits observed in non-pathological aging and Alzheimer's disease (AD) (Bartus et al., 1982; Drachman & Leavitt, 1974; McCleery et al., 2016; Montplaisir et al., 1995; Richter et al., 2014; Wisor et al., 2005; Xia et al., 2022). Given the abundance of evidence highlighting the role of the declining central cholinergic system in the cognitive symptoms associated with AD, the central cholinergic system has been a target for the treatment of the cognitive symptoms associated with AD.

Acetylcholinesterase inhibitors (AChEIs) are one of the few FDA-approved treatments for the cognitive impairments associated with AD, however they provide only moderate symptomatic benefit in clinical populations and display dose-limiting side effects due to non-specific action on peripheral muscarinic acetylcholine receptors (mAChRs) (Galimberti & Scarpini, 2016; Rogers et

al., 1998). The effects of acetylcholine signaling are mediated through the mAChRs and nicotinic acetylcholine receptors (nAChR). Due to the narrow therapeutic window and limited efficacy of AChEIs, there has been an effort to develop selective cholinergic ligands. Of these the five mAChRs (M_1-M_5), the M_1 mAChR is found postsynaptically in frontal cortical and hippocampal areas, and so is ideally placed to mediate cognition and arousal (Levey et al., 1991; Marino et al., 1998; Rouse et al., 1999; Spencer et al., 1986). Targeting of the M_1 mAChR has shown great promise in enhancing cognition in clinical trials, typified by the M_1/M_4-preferring agonist xanomeline, which progressed in development, showing trends toward the improvement of behavioral and cognitive deficits in AD. However, xanomeline failed in initial clinical trials due to its lack of selectivity resulting in dose-limiting adverse effects attributed to effects at peripheral M_2 and M_3 mAChRs (Bodick et al., 1997; Langmead et al., 2008). Currently, xanomeline compounded with the peripherally restricted muscarinic antagonist trospium is in clinical trials for the treatment of schizophrenia (Brannan et al., 2021) and psychosis in AD (ClinicalTrials.gov: NCT05511363). Due to the orthosteric binding site being highly conserved across the five mAChRs achieving sub-type selectivity has proved difficult (Bender et al., 2017), so alternative methods targeting less highly conserved allosteric sites have been pursued (Bubser et al., 2012; Conn et al., 2009; Jones et al., 2012)

The development of allosteric modulators, compounds that bind to topographically distinct and less highly conserved sites disparate to the orthosteric acetylcholine binding site (Christopoulos, 2002), has greatly improved the subtype selectivity of cholinergic ligands (Conn et al., 2009). This has led to the development of a number of potent M_1 positive allosteric modulators (PAMs) (Foster et al., 2014; Ghoshal et al., 2016; Rook et al., 2018; Uslaner et al., 2018) typified by the M_1 mAChR PAM tool compound VU0453595 (Ghoshal et al., 2016). These M_1 mAChR PAMs do not directly activate the receptor but potentiate the effects of ACh by altering the conformation of G-protein coupled receptors (GPCRs) to either enhance the affinity of acetylcholine for the orthosteric site or to enhance intracellular coupling to G-proteins. As M_1

mAChR PAMs do not have any intrinsic activity but enhancing the effects of endogenous acetylcholine signaling, these ligands provide a potential advantage over compounds with agonist activity by maintaining the physiologically relevant spatial and temporal endogenous signaling (Bubser et al., 2012; Conn et al., 2009).

Previous work by our group has demonstrated that the M_1 mAChR PAM VU0453595 enhances wakefulness and arousal in young rats and mice when dosed in the inactive phase, however effects on arousal in 22-26-month-old mice during the inactive were attenuated (Gould et al., 2020). As M_1 mAChR PAM efficacy is dependent on existing cholinergic signaling, and it has been shown that cholinergic signaling decreases with non-pathological aging in rodents (Mitsushima et al., 1996; Wu et al., 1988), we hypothesized this decrease in efficacy was due to the declining cholinergic function. ACh activity also varies across the circadian rhythm, with high levels of acetylcholine during the active phase and low levels during the active phase (Mitsushima et al., 1996). Considering this, we hypothesized that the M_1 mAChR PAM VU0453595 would display greater efficacy during the active phase in non-pathologically aged mice. As such, we characterize the effects of the M_1 mAChR PAM VU0453595 on sleep/wake architecture and arousal deficits at three ages of mice (3-4-, 19-20- and 26-28-months-old) across the circadian rhythm.

In the present study, we investigate whether the declining central cholinergic structure in non-pathological aging influences the efficacy of the M_1 mAChR PAM VU0453595. Here we assess age-related changes in cholinergic structure in the prefrontal cortex (PFC), an area richly innervated by cholinergic basal forebrain projections (M. M. Mesulam, 1990) which is vitally important in normal wake and arousal (B. E. Jones, 2020). These data provide the first evidence that in modestly aged mice (19-20-month-old), M_1 mAChR PAMs display wake and arousal-boosting properties during the active phase, while in contrast, in severely aged mice (26-28-month-old), M_1 PAM efficacy during the active phase is attenuated unless dosed in combination with the AChEI donepezil. However, anatomical studies utilizing ChAT IHC in the PFC reveal no

change in cholinergic structure across all ages tested. In addition, we demonstrate that the M_1 mAChR PAM VU0453595 displays no adverse effects in aged mice.

4.2 Methods

Subjects.

Young adult (3-4-month-old, n=28 for EEG, n=7 for IHC) and aged adult (19-20-month-old, n=14 for EEG and n=12 for side effect profiling, n=9 for IHC and 26-28-month-old, n=18 for EEG, and n=9 for IHC) male C57BL/6J wild type mice (Jackson Laboratories) were group-housed in 2-5 mice per cage prior to EEG surgery. Following surgery all animals were housed individually. For all studies, animals were housed in a temperature and humidity-controlled environment under a 12/12hr light dark with food and water available *ad libitum*. 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*.

Compounds.

VU0453595 (3-30 mg/kg) was synthesized in house and dissolved in 5% (2-Hydroxyypropyl)-beta-cyclodextrin, donepezil (3 mg/kg) (AstaTech inc, Bristol, PA) was dissolved in saline. All compounds were dosed at a volume of 10ml/kg via intraperitoneal (I.P.) injection.

Electroencephalography.

Surgery. EGG telemetry devices were implanted as previously described (Fisher et al., 2020; Gould et al., 2020). All animals were implanted with an HD-X02 telemetric transmitter (Data Sciences International [DSI], Minneapolis, MN) under isoflurane anesthesia. The transmitters were implanted subcutaneously off the midline of the dorsal flank. Two sets of biopotential transmitter leads tracked to the skull and neck. Holes were drilled in the skull (+1.5 mm, and -3 mm from Bregma, respectively and ± 2 mm contralateral to the midline). One set of biopotential leads were contacted with the dura and covered with dental cement (Patterson Dental, USA). The

remaining biopotential leads were placed in the nuchal muscle for electromyogram (EMG) recording.

Examining sleep/wake architecture and qEEG. EEG and EMG were recorded for 24-hours starting at the beginning of either the light period of the diurnal cycle (inactive phase) or the dark period of the diurnal cycle (active phase). Animals were dosed with VU0453595 (3.0 – 30 mg/kg I.P.), and/or donepezil (1.0-3.0 mg/kg I.P.) or appropriate vehicle 2 hours into the phase of interest. All recordings were sampled at a rate of 500Hz and transmitted via a receiver (RPC-1, DSI, MN) placed below the home cage. Each receiver was connected to a data exchange matrix (DSI, MN) which transferred the data to a computer which utilized Ponemah v3.0 (DSI) for recording.

Sleep Staging and analysis. All scoring was performed by trained observers blinded to age, compound, and dose. Neuroscore 3.3.1 software (DSI) was used to determine sleep/wake stages (wake, non-rapid eye movement (NREM) or rapid eye movement (REM) sleep) in 5-second epochs, based on characteristic EEG frequency and amplitude, and EMG activity as previously described by our group (Gould et al., 2016; Nedelcovych et al., 2015). The duration of time in each state (wake, NREM and REM), was assessed in 2 or 12 hr bins across each 24 hr period. Sleep bout analysis was performed by calculating the mean length of time spent in a state (wake or NREM sleep) per bout in the 8-hours following compound dosing, bout duration; and the number of times a mouse entered a state in the 8-hours following compound dosing, bout number. These served as primary variables to assess the effects of VU0453595 on sleep-wake architecture. All experiments are displayed in zeitgeber time, where ZT0 indicates transition from the dark (active) into the light (inactive) phase.

qEEG Spectral Power Analysis. Quantitative EEG (qEEG) relative power spectra were computed in 1 Hz bins from 0.5-80Hz using a Fast Fourier Transform with a Hamming window and overlap ratio of 0.5. Spectral power was examined within discrete states (Wake, NREM and REM). The power within each 1 Hz bin in each 5-second epoch within a given state was averaged,

to yield the state dependent relative spectral power as previously described(Gould et al., 2016; Nedelcovych et al., 2015). The dose dependent effects were determined within subject by expressing the power spectrum 1-2 hrs post dosing with respect to the comparable 1Hz interval to the baseline period in the 1-hr prior to dosing. For assessment of power change within a frequency band over time within wake and NREM sleep, spectral power was binned from 0.5-4Hz (delta), 4-8Hz (theta), 8-13Hz (alpha), 13-30Hz (beta) and 30-80Hz (gamma). This was averaged in 1-hr bins from 2-hrs pre dose until 8-hrs post dose and compared to a baseline 1-2 hrs after light change. For age-related comparisons the 1-hr pre-vehicle dosing baseline was used for comparison of the full spectrogram with the 19-20- and 26-28-month-old mice displayed relative to 3-4-month-old mice. For assessments within power band across time the time points at 19-20- and 26-28-months-old were displayed relative to the corresponding time in the young mice.

Immunohistochemistry

Sample collection. 7, 3-month-old; 9, 19-month-old; and 9, 26-month-old mice were perfused with 0.1M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) (both pH 7.35-7.45). Following perfusion brains are extracted and placed in 4% PFA solution overnight and then transferred to a 30% sucrose solution in 0.1 M phosphate buffer for cryoprotection. Brains were cut on a Leica sliding microtome to produce 45-µm-thick slices, with approximately 80 µm between slices. The sections were transferred to a storage solution containing 300 g sucrose and 300 mL ethylene glycol in 0.1 M phosphate buffer, pH 7.4 and were stored at -20° C until processing for immunohistochemistry.

Free-floating sections were first washed 6 times in 50 mM TBS for 10 minutes each followed by antigen retrieval which consisted of boiling sections for 10 minutes in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 90°C. Following another three washes in TBS, sections were incubated for twenty minutes in 50 mM TBS containing 4% normal horse serum and 0.2% Triton X-100. Sections were then incubated at 4° C for approximately 36 hours with a 1;100-dilution of a goat-anti acetylcholinetransferase (ChAT) antibody (Millipore, AB144P)

in 50 mM TBS containing 4% normal horse serum and 0.2% Triton X-100/) and 2 days at 4C. After six 10-min washes in 50 mM TBS, sections were incubated for two hours at room temperature with a Cy3-conjugated anti-goat antibody (Jackson Immunoresearch) diluted 1:1250 in TBS containing 4% normal horse serum and 0.2% Triton X-100 and then counter-stained with DAPI nuclear stain. Sections were washed six times for 10 minutes each with TBS for 10 minutes each and mounted from 0.15% gelatin onto Superfrost/Plus microscope slides (Fisher Scientific). Air dried sections were dehydrated in an ascending series of ethanol (3 minutes in 50% ethanol, 70% ethanol, and 3x 3min in 100% ethanol) followed by 2x 3 minutes in Histoclear and then cover slipped using DPX mounting medium (Electron Microscopy Services).

Image acquisition and analysis. A spinning disk confocal microscope (Nikon) was used to acquire a z-stack of composite images at three rostrocaudal levels of the medial prefrontal cortex at 20x magnification. ChAT fibers innervation was analyzed in a coronal mouse brain section at the approximate anterior to posterior level of AP +1.9 (Paxinos and Franklin, 2001). First, a 50-µm wide strip from the cortical surface to the white matter of the forceps minor of the corpus callosum was drawn over a composite image of the medial prefrontal cortex taken at 20x magnification. This strip was placed in the middle of the prelimbic area, corresponding to area 32 of Brodman (Laubach et al., 2018; le Merre et al., 2021; Vogt et al., 2013). The z-stack with the highest staining intensity of ChAT-like immunoreactivity (-li) was selected for subsequent image analysis. First, a 50-µm-wide strip encompassing cortical layers I-VI from the dura to the beginning of the forceps minor of the corpus callosum was overlaid to the image. NIS-Element's General Analysis (GA3) software module (Nikon) was then used to trace ChAT-like immunoreactive (-li) fibers within this outlined area to determine the length of ChAT-li fibers. The analysis parameters were empirically set in such a way that the majority, but not all ChAT-li fibers were traced. The fibers not identified by the automated tracing system were then manually traced by two independent individuals blinded to age group. Each rater calculated the total fiber length as the sum of the auto-traced fibers length plus the length of the manually traced fibers. The means of the total fiber length

obtained by the two raters was then divided by the area of the outlined strip to determine the fiber density (fiber length [µm]/area [µm^2]). Additionally, the fiber density in the superficial layers (I-III) and the deep layers (V-VI) as well as the cortical thickness were determined. All procedures from image acquisition to fiber tracing were performed by individuals that were blinded with regards to the age group of the animals. Tracings were done by two independent raters.

Non-conditioned behaviors and pharmacokinetics

Modified Irwin Test Battery. The effects of VU0453595 (30 mg/kg) on autonomic and somatomotor function in 19-20-month-old C57BL/6J mice were assessed utilizing the modified Irwin test battery (Irwin, 1968) as previously described by our group(Bubser et al., 2014). Assessments were performed 30, 60,120 and 240 min after i.p. administration of VU0453595 or vehicle. All behaviors were scored as 0- absent, 1-mild, or 2-severe. (See Table 4.1 for full list of behaviors assessed).

pK study. VU0453595 was formulated as previously described and dosed i.p. in 3-4- and 19-20-month-old C57BL/6J mice at 30 mg/kg. Brain and plasma were collected at 1-hour and 4-hours post dosing non-serially (n=4 mice per timepoint per age). Brain and plasma concentrations were quantified by electrospray ionization using an AB Sciex Q-TRAP 5500 (Foster City, CA) that was coupled to a Shimadzu LC-20AD pump (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a C18 column (3 x 50 mm, 3 mm; Fortis Technologies Ltd, Cheshire, UK) that was thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted); mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). A 10% B gradient was held for 0.2 minute and was linearly increased to 90% B over 0.8 minute, with an isocratic hold for 0.5 minutes, before transitioning to 10% B over 0.05 minute. The column was re-equilibrated (1 minute) before the next sample injection. The total run time was 2.55 minutes, and the HPLC flow rate was 0.5 ml/min. The source temperature was set at 500°C, and mass spectral analyses were performed using a Turbo-Ion

spray source in positive ionization mode (5.0-kV spray voltage) and using multiple-reaction monitoring of transitions specific for the analyte (m/z 323.2 to 189.4 at 30 eV). All data were analyzed using AB Sciex MultiQuant 2.2 software. The lower limits of quantitation for VU0453595 was determined at 0.5 ng/ml in plasma and in brain homogenates. Brain to plasma ratios (K_p) were calculated using concentrations 1hr post-dosing.

Statistical analysis

A repeated measures two-way analysis of variance (ANOVA) (repeated by both factors) with Dunnett's comparisons to the vehicle treated condition was applied to examine the effects of time and dose within each stage (wake, NREM, REM). To assess age-related differences in sleep state two-way repeated measures analysis of variance (repeated by one factor), with Sidak's corrections for multiple comparisons was used. Significance was defined as $p<0.05$. To assess the effects of dose and frequency or within a frequency band over time a repeated measures two-way ANOVA with Dunnett's multiple comparisons, or linear mixed effects model when there were missing data points, to the vehicle treated condition was utilized. To assess the effects of age and frequency a repeated measures two-way ANOVA with Dunnett's correction for multiple comparisons comparing to 3-4-month-old mice was used. All anatomical data were compared by one-way ANOVA followed by Dunnett's test. Significance was defined as $p<0.05$. For full statistics see Table 4.2.

4.3. Results

The M_1 mAChR PAM VU0453595 increased wakefulness in the active phase in aged mice.

When dosed in the active period in 3-4-month-old mice 30 mg/kg VU0453595 had no effect on duration of time in wake at any timepoint following post-hoc analysis (main effect of dose $p=0.0008$; and time $p<0.0001$; no dose x time interaction) or duration of time in NREM sleep (main

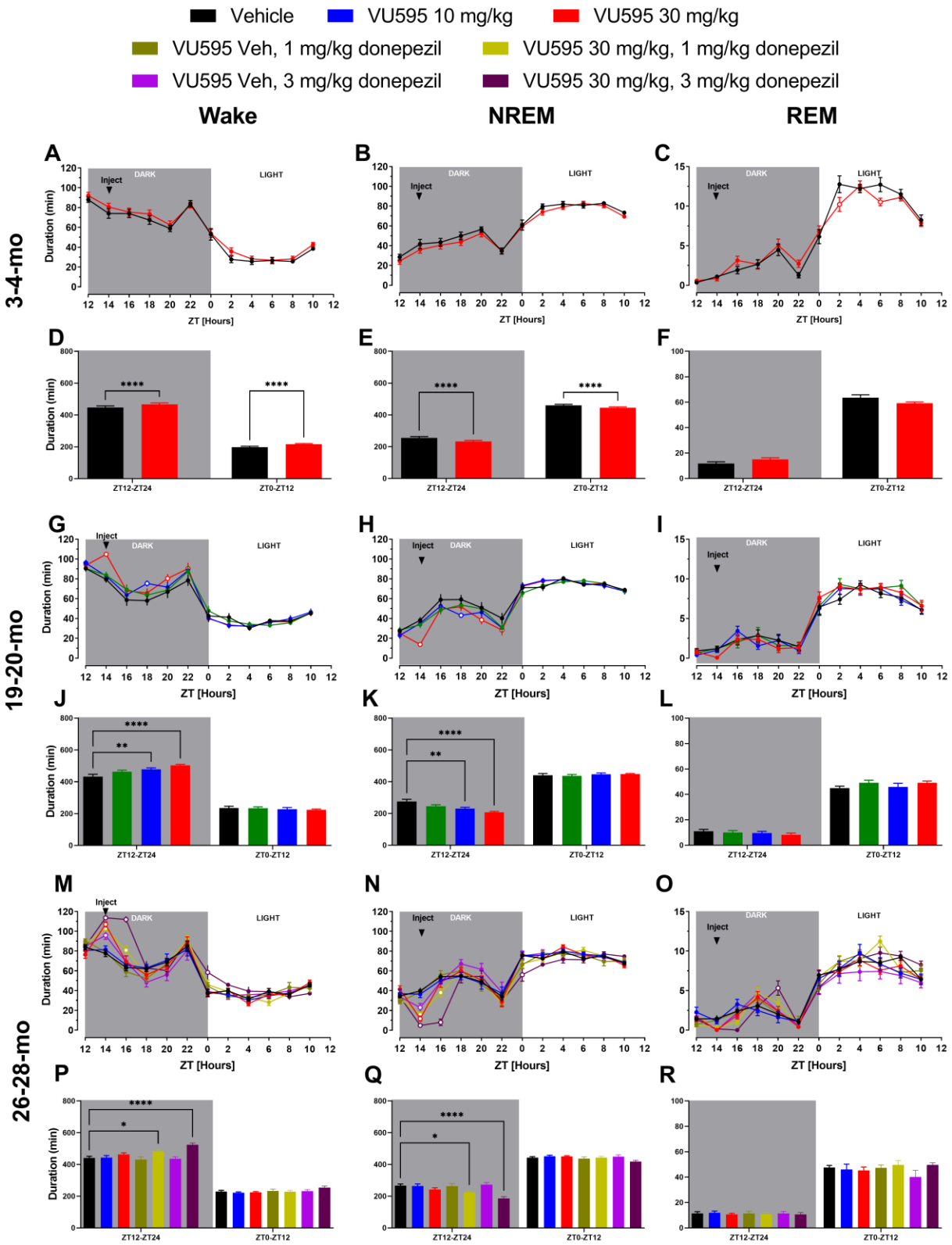
Vehicle
VU595 10 mg/kg
VU595 30 mg/kg
VU595 Veh, 1 mg/kg donepezil
VU595 30 mg/kg, 1 mg/kg donepezil
VU595 Veh, 3 mg/kg donepezil
VU595 30 mg/kg, 3 mg/kg donepezil
Wake
NREM
REM
3-4-mo
19-20-mo
26-28-mo
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Inject
DARK
LIGHT
Duration (min)
ZT [Hours]
ZT12-ZT24
ZT0-ZT12
****
**
*

Figure 4.1. VU0453595 increased wakefulness in the active phase in 3-4-month-old and 19-20-month-old mice, and in combination with donepezil in 26-28-month-old mice. Shown is the duration of time spent in wake (A, D, G, J, M, P), NREM sleep (B, E, H, K, N, Q) and REM sleep (C, F, I, L, O, R) in 3-4-month-old (A-F), 19-20-month-old (G-L) and 26-28-month-old (M-R) mice following VU0453595 +/- donepezil administration 2 hours into the active phase (see arrowhead). In 3-4-month-old mice VU0453595 has no effect on wake at any time point following dosing in the active phase (A), although an increase in total wake is observed from ZT12-ZT24 and ZT0-12 following 30 mg/kg VU0453595 (D). VU0453595 has no effect on NREM sleep at any time point following dosing in the active phase in 3-4-month-old mice (B), however decreased NREM sleep is seen following 30 mg/kg VU0453595 in the active phase (E). VU0453595 has no effect on REM sleep in the active phase following dosing, a decrease in REM sleep is seen at ZT2 and ZT 6 in the subsequent inactive phase (C). No effect is observed when assessing REM sleep duration in 12-hour bins (F). In 19-20-month-old mice 10 and 30 mg/kg VU0453595 dosing in the active phase produced increased wake seen in 2-hr bins (G) and 12-hr bins (J). Conversely 10 and 30 mg/kg VU0453595 reduced NREM sleep following dosing in the active phase in 19-20-month-old mice when assessed in 2-hr bins (H) or 24-hr bins (K). VU0453595 had no effect on REM sleep when dosed in the active phase in 19-20-month-old-mice (I, L). In 26-28-month-old mice, 30 mg/kg VU0453595 dosed alone produced a transient increase in wake, while dosing in combination with 1 and 3 mg/kg donepezil produced more robust increases in wakefulness than with either compound alone (M) when assessed in 12-hr bins neither VU0453595 or donepezil dosed alone produced any effect, however 30 mg/kg VU0453595 dosed with 1 and 3 mg/kg donepezil produced increased wake (P). 30 mg/kg VU0453595 produced reduced NREM sleep when dosed alone, with greater effects seen in conjunction with 1 and 3 mg/kg donepezil (Q). 30 mg/kg VU0453595 dosed in combination with 3 mg/kg donepezil produced an increase in REM sleep at ZT20 following dosing (O), no effects were seen at any dose in 12-hr bins (R). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I, M-O) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA or RM mixed effect model matching by both factors followed by Sidak's (A-C) or Dunnett's test (G-I, M-O)), or 12-hour bins (D-F, J-L, P-R) * indicates $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ compared to vehicle (RM 1-way ANOVA or RM mixed effect model followed by Sidak's (D-F) or Dunnett's test (J-L, P-R), n=11-14/group; See table 4.2 for full statistical analysis.

effect of dose p=0.0008; and time p<0.0001; no dose x time interaction) (Figure 4.1A and B). When assessing in 24-hr bins, increased wake was observed from ZT12-24 and ZT0-12 (main effect of dose p=0.0008, and time p<0.0001, but no dose x time interaction) (Figure 4.1D), with decreased NREM sleep observed from ZT 12-24 (main effect of dose p=0.0008; and time p<0.0001; no dose x time interaction) (Figure 4.1E). 3-4-month-old mice displayed no effect on time in REM sleep immediately following dosing with 30 mg/kg VU0453595, with a reduction seen 12 and 16 hours after dosing (no main effect of dose; main effect of time p<0.0001; dose x time interaction p=0.0044) (Figure 4.1C). No effect on time in REM sleep was observed when assessing in 12-hr bins (no main effect of dose; main effect of time p<0.0001; dose x time interaction p=0.0208) (Figure 4.1F).

In the active period in 19-20-month-old mice 30 mg/kg VU0453595 produced an increase in wake duration following dosing, and 10 mg/kg produced a modest increase 4hrs after dosing (main effect of dose p=0.0062; time p<0.0001; and time x dose interaction p=0.0007) with

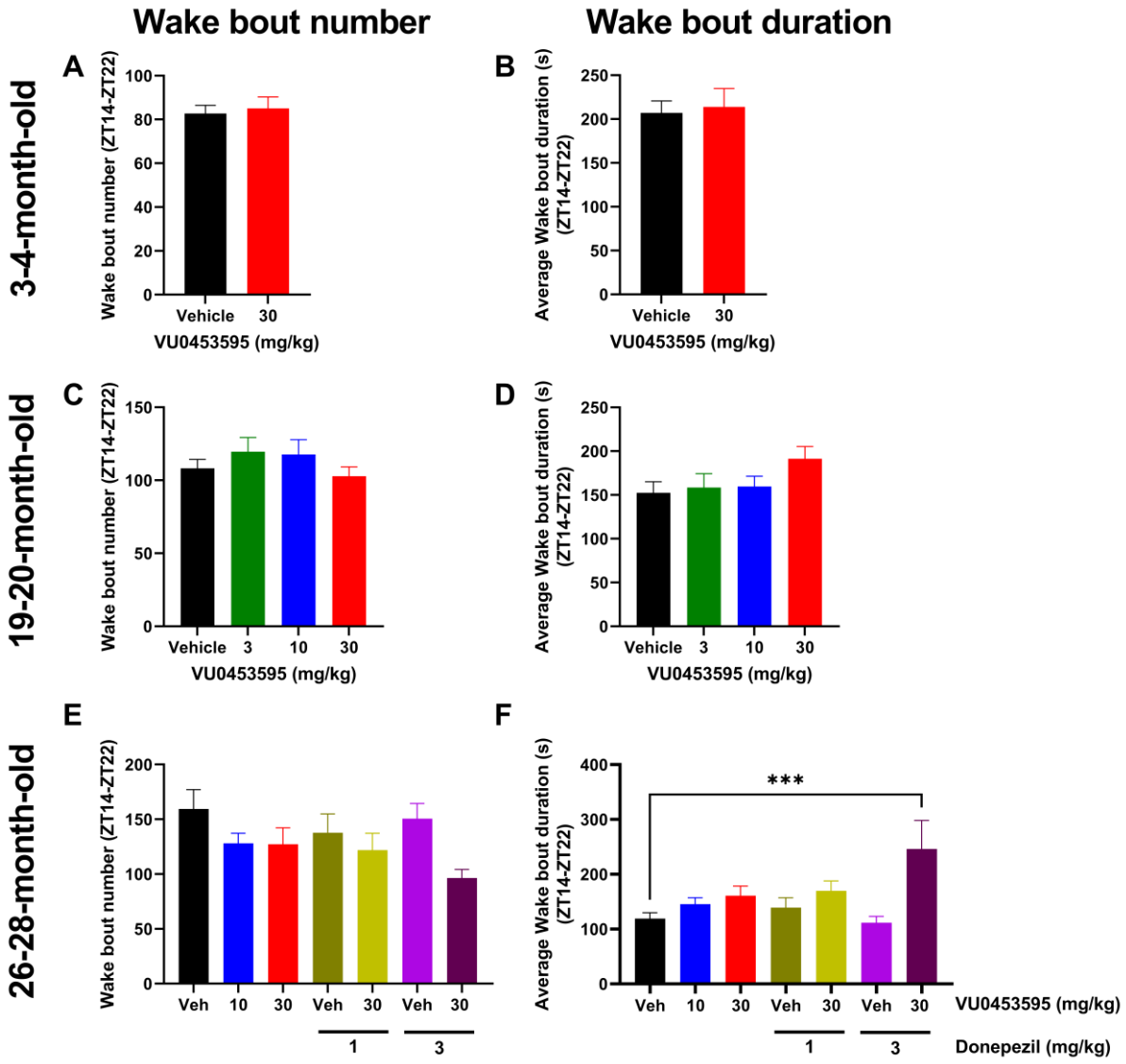

Figure 4.2. VU0453595 in combination with donepezil increased wake bout number during the active phase in 19-20-month-old mice. Shown is the average wake bout number (A, C, E) and the average wake bout duration (B, D, F) 3-4-month-old (A, B), 19-20-month-old (C, D) and 26-28-month-old mice (E, F) mice for 8 hours following dosing in the active phase. In 3-4-month-old mice VU0453595 dosed in the active phase has no effect on wake bout number (A) or wake bout duration (B). In 19-20-month-old mice VU0453595 dosed in the active phase has no effect on wake bout number (C) or wake bout duration (D). In 26-28-month-old mice VU0453595 dosed alone or in combination with donepezil in the active phase has no effect on wake bout number (E). 30 mg/kg VU0453595 dosed in combination with 3 mg/kg donepezil increased wake bout duration (F). Data are expressed as overall means ± S.E.M., n=11-14/group. *** indicates p<0.001 compared to vehicle (RM 1-way ANOVA or RM mixed effect model followed by Dunnett's test). See table 4.2 for full statistical analysis.

decreased NREM sleep duration at the same doses (main effect of dose p=0.0021; time p<0.0001; and dose x time p=0.0003) (Figure 4.1G and H). When assessing in 12-hr bins, 10 and 30 mg/kg VU0453595 increased wake duration from ZT12-24 (main effect of dose p=0.0062; time

p<0.0001; and time x dose interaction p=0.0011) (Figure 4.1J) and reduced NREM sleep duration from ZT12-24 (main effect of dose p=0.0021; time p<0.0001; and dose x time p=0.0003) (Figure 4.1K). When dosed in the active phase VU0453595 had no effect on REM sleep when assessed in 2-hr bins or 12-hr bins (both, no main effect of dose; main effect of time p<0.0001; no dose x

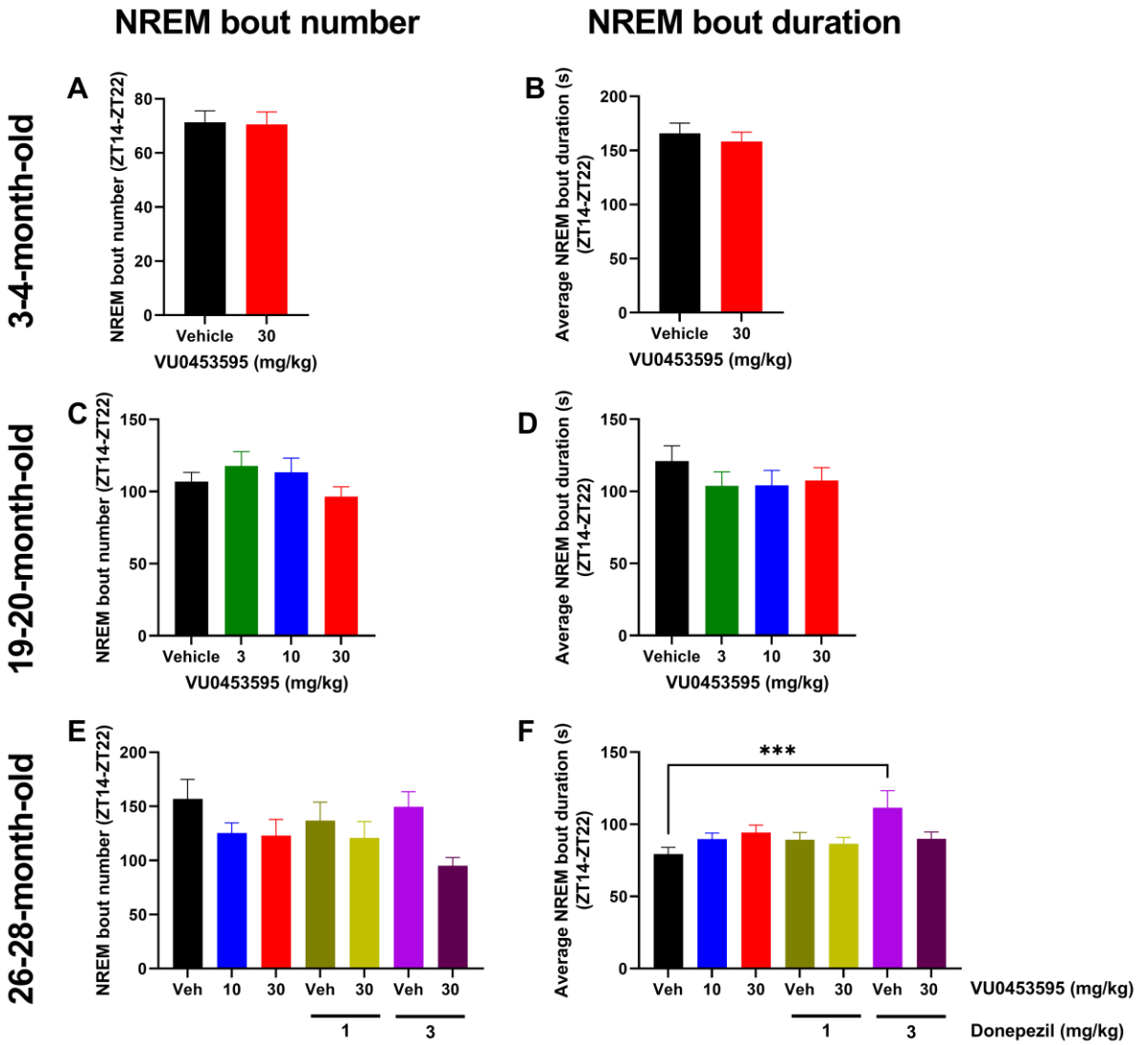

Figure 4.3. VU0453595 in combination with donepezil increased wake bout number during the active phase in 19-20-month-old mice. Shown is the average wake bout number (A, C, E) and the average wake bout duration (B, D, F) 3-4-month-old (A, B), 19-20-month-old (C, D) and 26-28-month-old mice (E, F) mice for 8 hours following dosing in the active phase. In 3-4-month-old mice VU0453595 dosed in the active phase has no effect on wake bout number (A) or wake bout duration (B). In 19-20-month-old mice VU0453595 dosed in the active phase has no effect on wake bout number (C) or wake bout duration (D). In 26-28-month-old mice VU0453595 dosed alone or in combination with donepezil in the active phase has no effect on wake bout number (E). 30 mg/kg VU0453595 dosed in combination with 3 mg/kg donepezil increased wake bout duration (F). Data are expressed as overall means ± S.E.M., n=11-14/group. *** indicates p<0.001 compared to vehicle (RM 1-way ANOVA or RM mixed effect model followed by Dunnett's test). See table 4.2 for full statistical analysis.

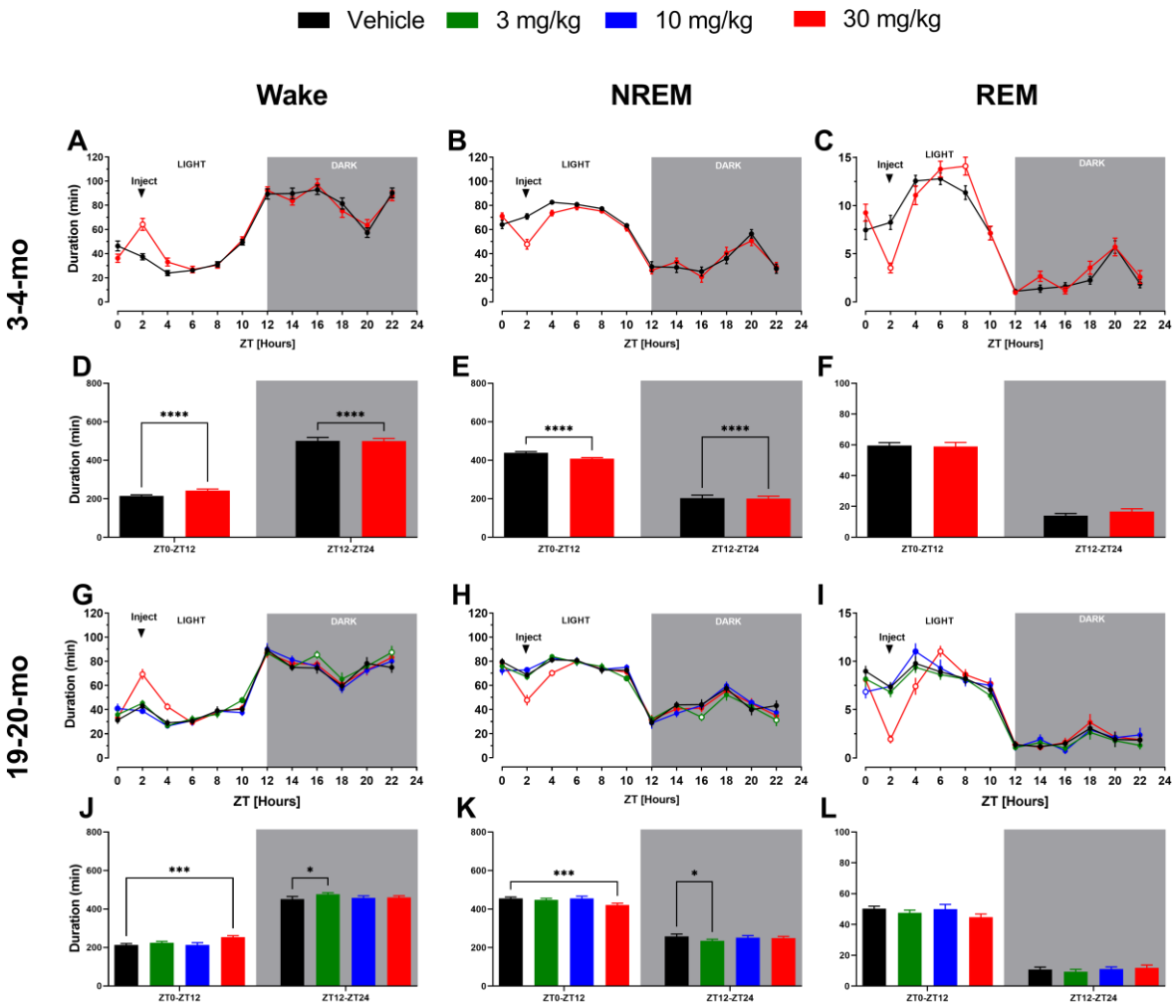

Figure 4.4. VU0453595 increased wakefulness in the inactive phase in young and aged mice in the inactive phase. Shown is the duration of time spent in wake (A, D, G, J), NREM sleep (B, E, H, K) and REM sleep (C, F, I, L) in 3-4-month-old (A-F) and 19-20-month-old (G-L) mice following VU0453595 administration 2 hours into the inactive phase (see arrowhead). In 3-4-month-old mice VU0453595 produced transiently increased wake following dosing in the inactive phase (A), with an increase in total wake from ZT0-12 following 30 mg/kg VU0453595 (D). VU0453595 produced decreased NREM sleep following dosing in the inactive phase in 3-4-month-old mice (B), with decreased NREM sleep is seen following 30 mg/kg VU0453595 from ZT0-12 (E). 30 mg/kg VU0453595 produced initially decreased REM sleep in the inactive phase following dosing, followed by a rebound increase seen at ZT10 (C). No effect is observed when assessing REM sleep duration in 12-hour bins (F). In 19-20-month-old mice 30 mg/kg VU0453595 dosing in the inactive phase produced increased wake seen in 2-hr bins (G) and 12-hr bins (J), while following dosing with 3 mg/kg VU0453595 in the inactive phase increased wake was observed at ZT16 and 22 (G) with increased wake observed from ZT12-24 (J). Conversely 30 mg/kg VU0453595 reduced NREM sleep following dosing in the inactive phase in 19-20-month-old mice when assessed in 2-hr bins (H) or 24-hr bins (K), and 3 mg/kg produced reductions in NREM sleep at ZT 16 and 22 (H), with decreased NREM observed from ZT 12-24 (K). 30 mg/kg VU0453595 produced a transient decrease in REM sleep followed by a rebound increase when dosed in the inactive phase in 19-20-month-old-mice (I, L). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA matching by both factors followed by Sidak's (A-C) or Dunnett's test (G-I)), or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to vehicle (RM 1-way ANOVA followed by Sidak's (D-F) or Dunnett's test (J-L), n=13-14/group; See table 4.2 for full statistical analysis.

time interaction) (Figure 4.1I and L).

In 26-28-month-old mice 30 mg/kg VU0453595 produced increased time in wake following dosing, which was also observed following combination dosing with 1 mg/kg donepezil and an enhanced duration of effect following combination dosing with 3 mg/kg donepezil. 3 mg/kg donepezil alone produced a transient increase in wake duration (main effect of dose; time; and dose x time interaction, all $p<0.0001$) (Figure 4.1M), and at the same doses decreased NREM sleep duration (main effect of dose; time; and dose x time interaction, all $p<0.0001$) (Figure 4.1N). When assessing in 12-hour bins, only 30 mg/kg VU0453595 dosed with 1 or 3 mg/kg donepezil produced increased wake duration at ZT12-24 (main effect of dose, $p<0.0001$; time, $p<0.0001$; and dose x time interaction, $p=0.0056$) (Figure 4.1P), and at the same doses produced decreased NREM sleep duration (main effect of dose, $p<0.0001$; time, $p<0.0001$; and dose x time interaction, $p=0.0319$) (Figure 4.1Q). VU0453595 dosed alone produced no effect on REM sleep, however when dosed in combination with 3 mg/kg donepezil an increase in REM sleep was observed at ZT20 (no main effect of dose; main effect of time, $p<0.0001$; and a dose x time interaction, $p<0.0001$) (Figure 4.1O), no effect was observed when assessing in 12-hr bins (no effect of dose; or dose x time interation; main effect of time, $p<0.0001$).

The M_1 mAChR PAM VU0453595 dosed in combination with donepezil increased wake bout duration during the active phase in 26-28-month-old mice.

To assess the effects on wake fragmentation during the active phase we next investigated dose related effects on wake and NREM bout number and duration. VU0453595 dosed alone had no effect on wake bout number or wake bout duration at any age tested (Figure 4.2A-F). In 26-28-month-old mice, 30 mg/kg VU0453595 dosed in combination with 3 mg/kg donepezil increased wake bout duration, neither compound alone produced any effect on wake bout duration (main effect of dose, $p=0.0018$) (Figure 4.2F). No dose of VU0453595 tested produced an effect on NREM sleep bout number or NREM sleep bout duration at any age tested (Figure 4.3A-F). 3

mg/kg donepezil dosed alone in 26-28-month-old mice increased NREM sleep bout duration (main effect of dose, p=0.0008) (Figure 4.3F).

The M_1 mAChR PAM VU0453595 increased wakefulness in the inactive phase in all ages of mice tested.

In young mice when dosed in the inactive period 30 mg/kg VU0453595 increased wake (no main effect of dose; main effect of time p<0.0001; and dose x time interaction p<0.0001) and

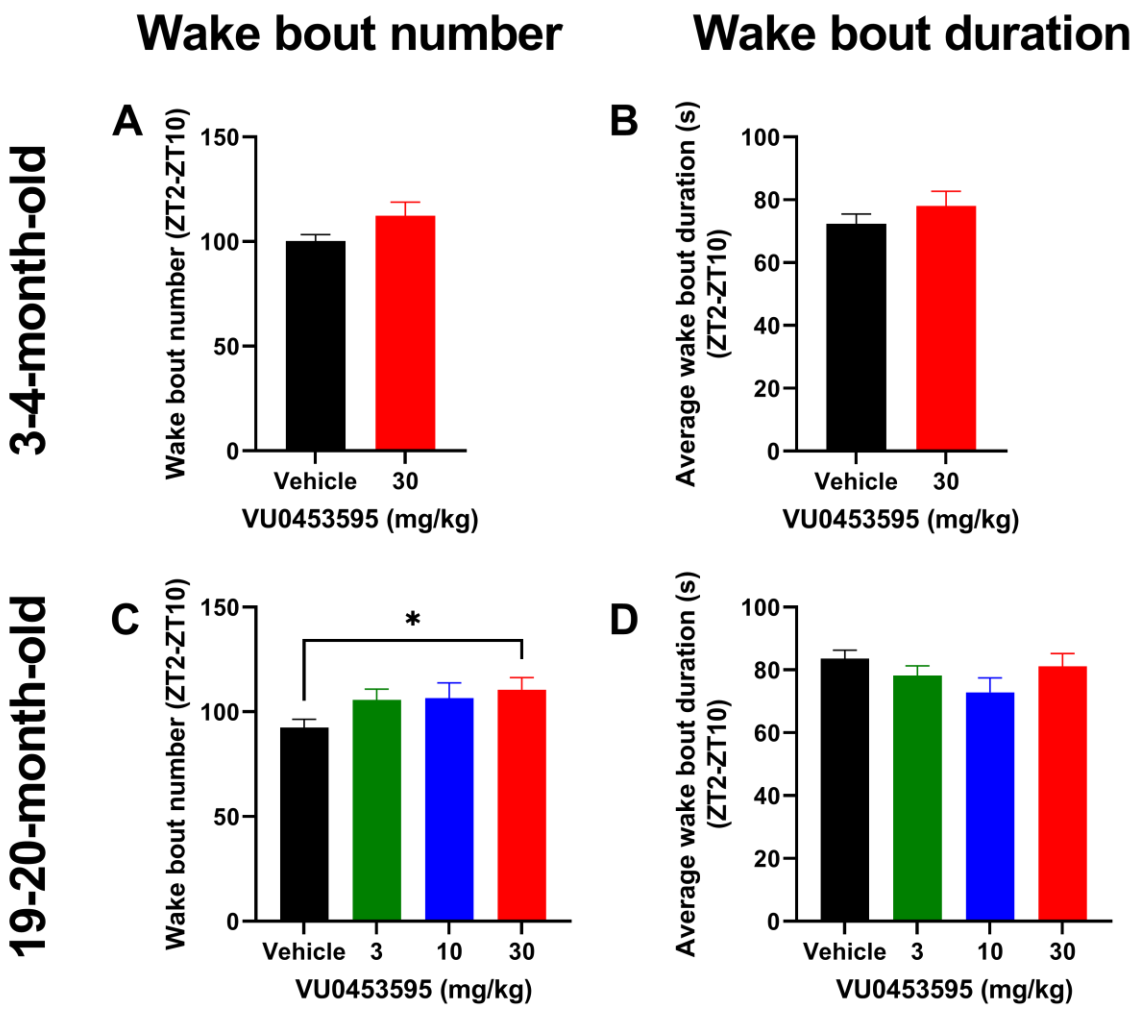

Figure 4.5. VU0453595 increased wake bout number during the inactive phase in 19-20-month-old mice. Shown is the average wake bout number (A, C) and the average wake bout duration (B, D) 3-4-month-old (A, B) and 19-20-month-old (C, D) mice for 8 hours following dosing in the inactive phase. In 3-4-month-old mice VU0453595 dosed in the inactive phase has no effect on wake bout number (A) or wake bout duration (B). In 19-20-month-old mice 30 mg/kg VU0453595 dosed in the inactive phase produced increased wake bout number (C) with no effect on wake bout duration (D). Data are expressed as overall means ± S.E.M., n=13-14/group. * indicates p<0.05 compared to vehicle (RM 1-way ANOVA followed by Dunnett's test). See table 4.2 for full statistical analysis.

reduced NREM sleep following dosing (no main effect of dose; main effect of time p<0.0001; and dose x time interaction p<0.0001) (Figure 4.4A and B). When assessing duration of time in wake in 12-hr bins, increased wake (no main effect of dose; main effect of time p<0.0001; and dose x time interaction p=0.0390) and decreased NREM sleep (no main effect of dose; main effect of time p<0.0001; and dose x time interaction p=0.0215) was observed from ZT0-12 following dosing

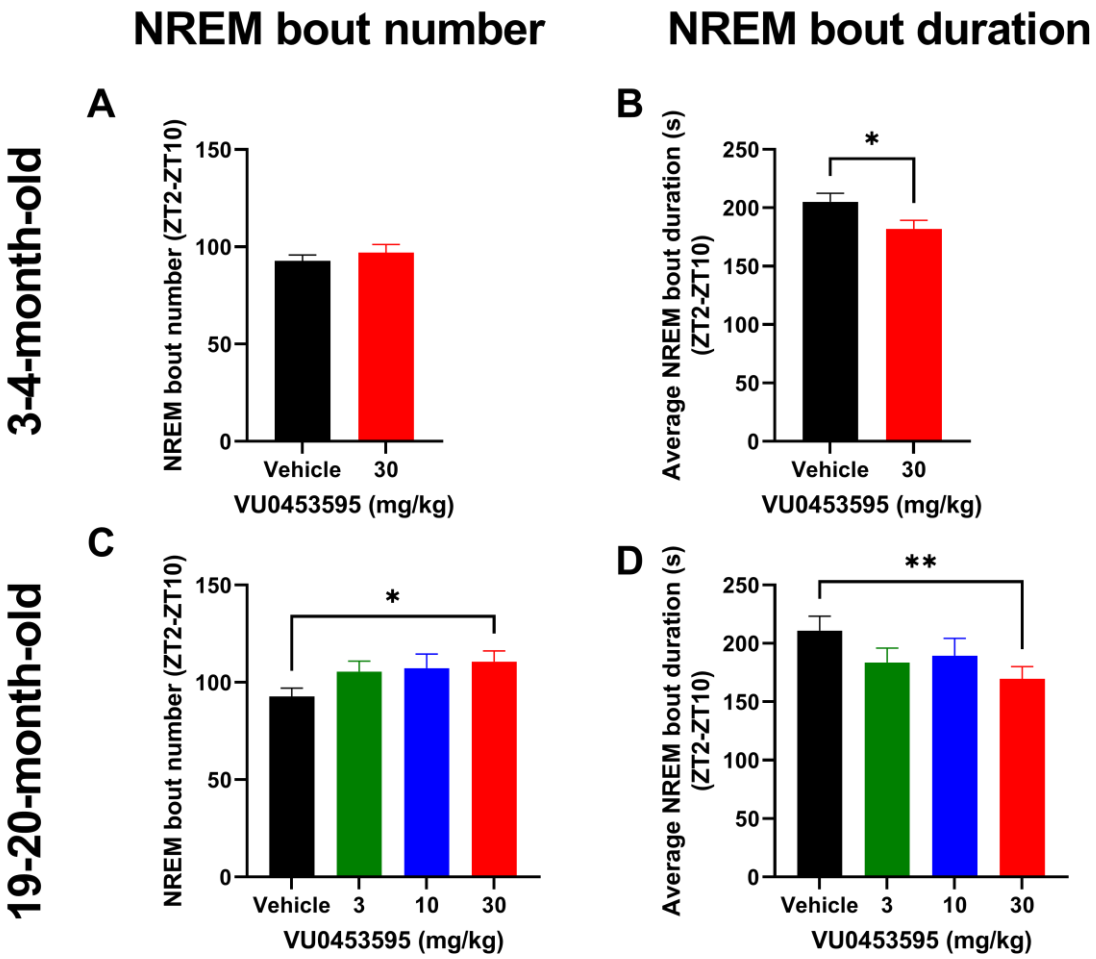

Figure 4.6. VU0453595 increased NREM sleep bout number and reduced NREM sleep bout duration during the inactive phase in 19-20-month-old mice. Shown is the average NREM sleep bout number (A, C) and the average NREM sleep bout duration (B, D) 3-4-month-old (A, B) and 19-20-month-old (C, D) mice for 8 hours following dosing in the inactive phase. In 3-4-month-old mice VU0453595 dosed in the inactive phase has no effect on NREM sleep bout number (A) but reduced NREM sleep bout duration (B). In 19-20-month-old mice 30 mg/kg VU0453595 dosed in the inactive phase produced increased NREM sleep bout number (C) with reduced NREM sleep bout duration (D). Data are expressed as overall means ± S.E.M., n=13-14/group. * indicates p<0.05, ** p<0.01 compared to vehicle (RM 1-way ANOVA followed by Dunnett's test). See table 4.2 for full statistical analysis.

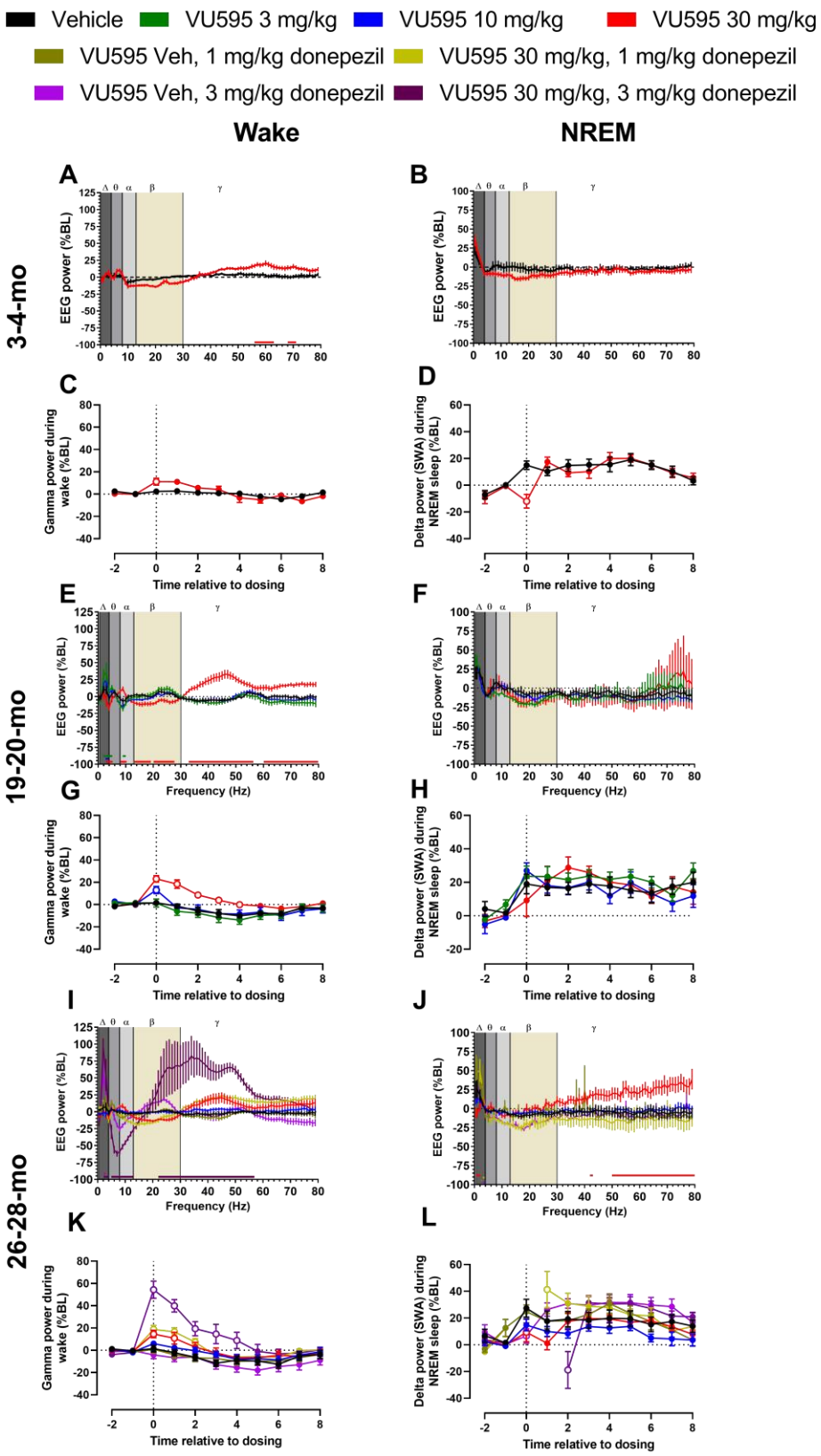
Vehicle
VU595 3 mg/kg
VU595 10 mg/kg
VU595 30 mg/kg
VU595 Veh, 1 mg/kg donepezil
VU595 30 mg/kg, 1 mg/kg donepezil
VU595 Veh, 3 mg/kg donepezil
VU595 30 mg/kg, 3 mg/kg donepezil
Wake
NREM
3-4-mo
19-20-mo
26-28-mo
A
B
C
D
E
F
G
H
I
J
K
L
EEG power (%BL)
Gamma power during wake (%BL)
Delta power (SWA) during NREM sleep (%BL)
Time relative to dosing
Frequency (Hz)

Figure 4.7. VU0453595 increased gamma power alone in 19-20-month-old mice and in combination with donepezil in 26-28-month-old mice in the active phase during wake. Shown is the relative spectral power during wake (A, E, I) and NREM sleep (B, F, J) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline, gamma power during wake across the active phase (C, G) and relative delta power (SWA) during NREM sleep across the active phase (D, H), during the active phase in 3-4- (A-D), 19-20-month-old (E-H) and 26-28-month-old (I-L) mice. In 3-4-month-old mice, during wake epochs 30 mg/kg VU0453595 increased gamma power at 52-52 and 68-71Hz (A) with a transient increase in total gamma immediately following dosing (C). VU0453595 produced no dose related effect on relative spectral power during NREM sleep in the 1-2-hrs following dosing (B), a transient decrease in delta power (SWA) during NREM sleep across the active phase in young mice was observed (D). In 19-20-month-old mice 30 mg/kg VU0453595 increased alpha and gamma power and reduced delta power during wake epochs (E) with a dose dependent increase in total gamma power observed (G). During NREM sleep VU0453595 produced no effect on spectral power (F), with no change in delta power (SWA) during NREM sleep observed (H). In 26-28-month-old mice VU0453595 and donepezil alone produced no significant effect on spectral power during wake, however 30 mg/kg VU0453595 in combination with 3 mg/kg donepezil produced in increase in delta and gamma powers, with a reduction in theta and alpha powers (I), with an increase in total gamma power seen following 30 mg/kg VU0453595 alone and with 1 and 3 mg/kg donepezil (K). VU0453595 increased gamma frequencies and reduced delta frequencies, 1 and 3 mg/kg donepezil increased relative power at 3Hz, while 30 mg/kg VU0453595 dosed with 1 mg/kg donepezil increased relative power at 2Hz (J). Delta power (SWA) during NREM sleep was decreased following dosing with 30 mg/kg VU0453595 alone and in combination with 3 mg/kg donepezil, while in combination with 1 mg/kg donepezil a transient increase was observed (L). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A, B, E, F, I, J) and means ± S.E.M. in 1-hour bins. (C, D, G, H, K, L), n=7-17/group, all time points in time courses contain n=7-17 mice (C, D, G, H, K, L). Groups with fewer than 11 mice are due to not all mice displaying NREM sleep n=11-17 mice tested per condition. Solid bars indicate p<0.05 compared to vehicle (A, B, E, F, I, J), open symbols indicate p<0.05 compared to vehicle (C, D, G, H, K, L) (RM 2-way ANOVA or RM mixed effect model matching by both factors followed by Sidak's (A-D) or Dunnett's test (E-L)). See table 4.2 for full statistical analysis.

with 30 mg/kg VU0453595 (Figure 4.4D and E). REM sleep transiently decreased, before then increasing following dosing with 30 mg/kg VU0453595 in the inactive phase (no main effect of dose; main effect of time p<0.0001; and dose x time interaction p<0.0001) (Figure 4.4C). When assessing in 12-hr bins, no effect on REM sleep was observed (no effect of dose; or dose x time interaction; main effect of time, p<0.0001) (Figure 4.4F).

In the inactive period in aged mice, 30 mg/kg VU0453595 produced an increase in wake following dosing, (main effect of dose p=0.0073; time p<0.0001; and time x dose interaction p<0.0001) with decreased NREM sleep at 30 mg/kg (main effect of dose p=0.0173; time p<0.0001; and dose x time interaction p<0.0001) (Figure 4.2E and F). When assessing in 12-hr bins, 30 mg/kg VU0453595 increased wake, and reduced NREM sleep from ZT0-12, while wake was increased and NREM sleep reduced at ZT12-24 following dosing with 3 mg/kg VU0453595 (wake: main effect of dose p=0.0073; time p<0.0001; and time x dose interaction p<0.0001 and NREM sleep: main effect of dose p=0.0173; time p<0.0001; and dose x time interaction p=0.0048)

(Figure 4.4J and K). In aged mice dosed in the inactive period, VU0453595 produced an initial reduction, followed by an increase in REM sleep (no main effect of dose, main effect of time $p<0.0001$, dose x time interaction $p<0.0001$) (Figure 4.4I). When assessing REM sleep duration in 12-hr epochs VU0453595 produced no effect in 19-20-month-old mice (Figure 4.4L).

The M_1 mAChR PAM VU0453595 increased wake and NREM sleep bout number in 19-20-month-old mice and decreased NREM bout duration in in 3-4- and 19-20-month-old mice during the inactive phase.

VU0453595 had no effect on wake bout number or wake bout duration in 3-4-month-old mice (Figure 4.5A and B). In 19-20-month-old mice VU0453595 increased wake bout number (main effect of dose, $p=0.0325$) (Figure 4.5C), with no effect on wake bout duration (Figure 4.5D). VU0453595 had no effect on NREM sleep bout number in 3-4-month-old mice (Figure 4.6A), but reduced NREM sleep bout length (main effect of dose, $p=0.0470$) (Figure 4.6B). In 19-20-month-old mice 30 mg/kg VU0453595 increased NREM sleep bout number (main effect of dose, $p=0.0321$) (Figure 4.6C) and reduced NREM sleep bout duration (main effect of dose, $p=0.0130$) (Figure 4.6D).

The M_1 mAChR PAM VU0453595 increased arousal in 19-20-month-old mice, and in combination with donepezil in 26-28-month-old mice during the active phase.

Modestly increased gamma power from 56-62 and 68-71Hz during wake epochs were seen during the 1-2 hours following dosing in 3-4-month-old mice dosed with 30 mg/kg VU0453595 (no main effect of dose; main effect of frequency $p<0.0001$; and dose x frequency interaction $p<0.0001$) (Figure 4.7A), when assessing gamma power across the active phase a transient increase in gamma power was observed at time point 0 following dosing (no main effect of dose; main effect of time $p<0.0001$; and dose x time interaction $p=0.001$) (Figure 4.7C). Furthermore, decreased beta power is observed (Figure 4.8). During NREM sleep in the 1-2 hours following dosing, no effect on spectral power was observed at any frequency on post hoc analysis (no main effect of dose; main effect of frequency $p<0.0001$; and dose x frequency interaction

p<0.0001) (Figure 4.7B). Delta power (SWA) during NREM sleep decreased at dosing time 0 (no main effect of dose; main effect of time p<0.0001; and dose x frequency time p<0.0001) (Figure 4.7D). Additionally, transiently increased theta, beta and gamma powers are observed (Figure 4.9).

In the 1-2 hours following dosing in the active phase 30 mg/kg VU0453595 increased

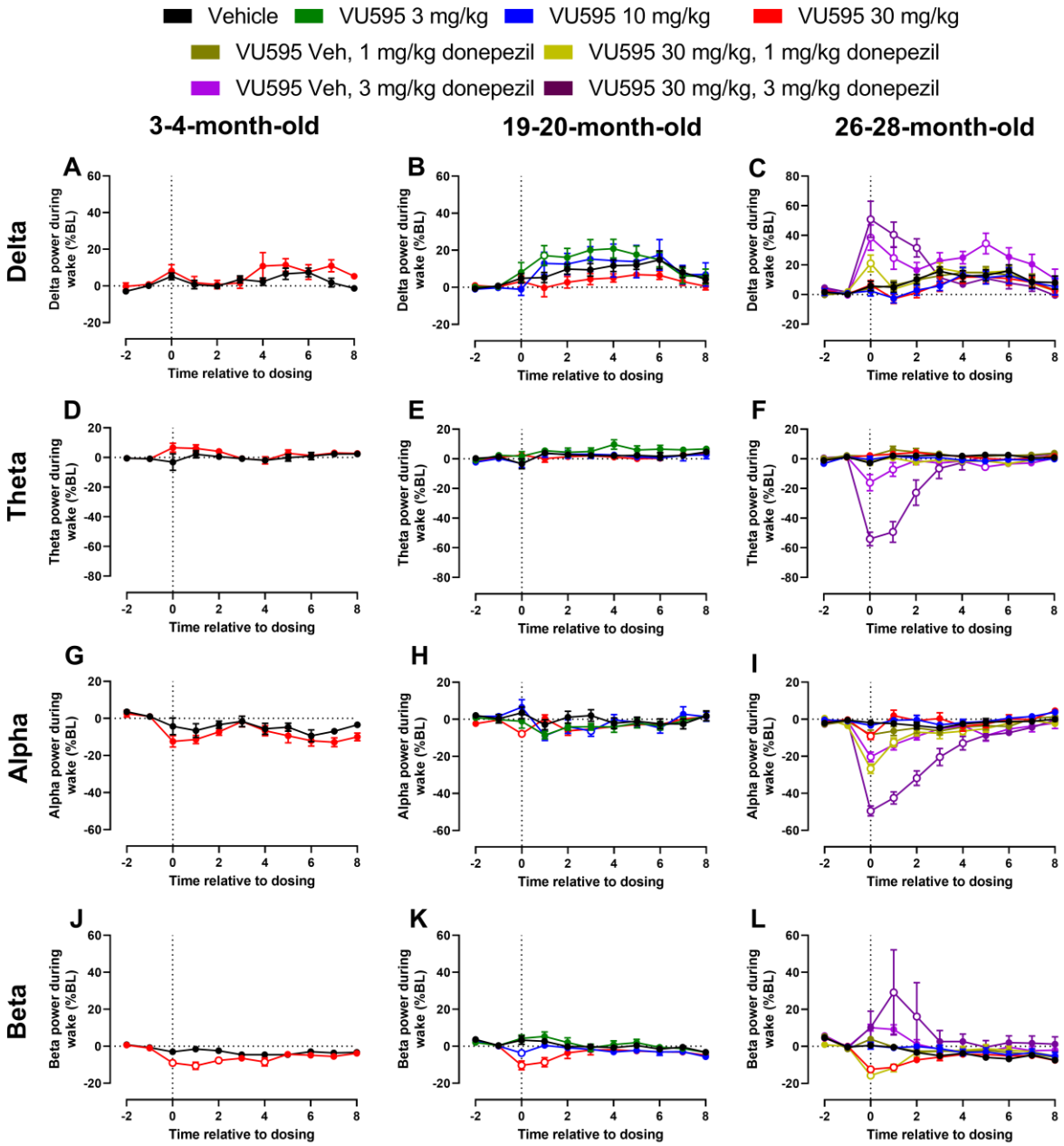

Figure 4.8. Time dependent effects of VU0453595 +/- donepezil on spectral power bands during wake in the active phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B, C), theta (D, E, F), alpha (G, H, I), and beta (J, K, L) power bands in 3-4-month-old (A, D, G, J), 19-20-month-old (B, E, H, K) and 26-28-month-old (C, F, I, L) mice during wake epochs in the active phase following VU453595 dosing at time 0. VU0453595 dosed in the active period produced no effect on delta power during wake in 3-4-month-old mice (A) or 26-28-month-old mice (C), with a transient increase observed following 3 mg/kg VU0453595 in 19-20-month-old mice (B). 3 mg/kg donepezil dosed with or without 3 mg/kg VU0453595 produced increased delta power (C). VU0453595 produced no effect on theta power at any age test (D, E, F), 3 mg/kg donepezil produced a reduction in theta power, while 3 mg/kg donepezil dosed with 30 mg/kg VU0453595 produced a longer reduction in theta power of greater magnitude (F). VU0453595 produced no effect on alpha power in 3-4-month-old mice (G), with modest reductions observed following 30 and 10 mg/kg VU0453595 dosing in 19-20-month-old mice (H) and 30 mg/kg dosing in 26-28-month-old mice. Donepezil dosed alone at 3 mg/kg, and in combination with 30 mg/kg VU0453595 at 1 and 3 mg/kg produced reduced alpha power (I). VU0453595 produced reduced beta power following dosing in 3-4-, 19-20- and 26-28-month-old mice (J, K, L). In 26-28-month-old mice this was also seen following 30 mg/kg VU0453595 dosing with 1 mg/kg donepezil, when dosed in combination with 3 mg/kg donepezil an increase in beta power was observed (L). Data are expressed as means ± S.E.M. in 1-hour bins, n=11-17/group, open symbols indicate p<0.05 compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 4.2 for full statistical analysis.

power during wake across the gamma range in 19-20-month-old mice (main effect of dose $p<0.0001$, frequency $p<0.0001$ and dose x frequency interaction $p<0.0001$) (Figure 4.7E), with a dose-dependent increase in total gamma observed with 30 mg/kg VU0453595 increasing gamma power for 4 hours following dosing (main effect of dose, $p=0.0063$; time, $p<0.0001$; and dose x time interaction, $p<0.0001$) (Figure 4.7G). Additionally, dose-related decreased beta and alpha power are observed (Figure 4.8). During NREM sleep in 19-20-month-old mice no effect of VU0453595 on spectral power is observed in the 1-2 hours following dosing (no main effect of dose; main effect of frequency, $p<0.0001$; and no effect dose x frequency interaction) or when assessing total delta power (SWA) during NREM sleep across time (no main effect of dose; main effect of time, $p<0.0001$; and no effect dose x time interaction). Consistent with this no changes in power are observed in any frequency band following dosing (Figure 4.9).

In 26-28-month-old mice VU0453595 or donepezil dosed alone produced no significant effect on spectral power in the 1-2 hours following dosing, however 3 mg/kg donepezil dosed with 30 mg/kg VU0453595 produced a robust increase in beta and gamma frequencies, with decreased theta, alpha and increased delta powers also observed (main effect of dose, $p=0.0081$; frequency, $p<0.0001$; and dose x frequency interaction, $p<0.0001$) (Figure 4.7I). When assessing total gamma power following dosing in the active phase a modest increase in gamma power was

observed following 30 mg/kg VU0453595 dosing alone or in combination with 1 mg/kg donepezil, with a more robust, prolonged increase observed following dosing with 30 mg/kg VU0453595 with 3 mg/kg donepezil (main effect of dose, $p<0.0001$; time, $p<0.0001$; and dose x time interaction, $p<0.0001$) (Figure 4.7K). Consistent with the findings 1-2 hours after dosing, increased delta and beta powers are observed, with decreased theta and alpha following dosing with 3 mg/kg

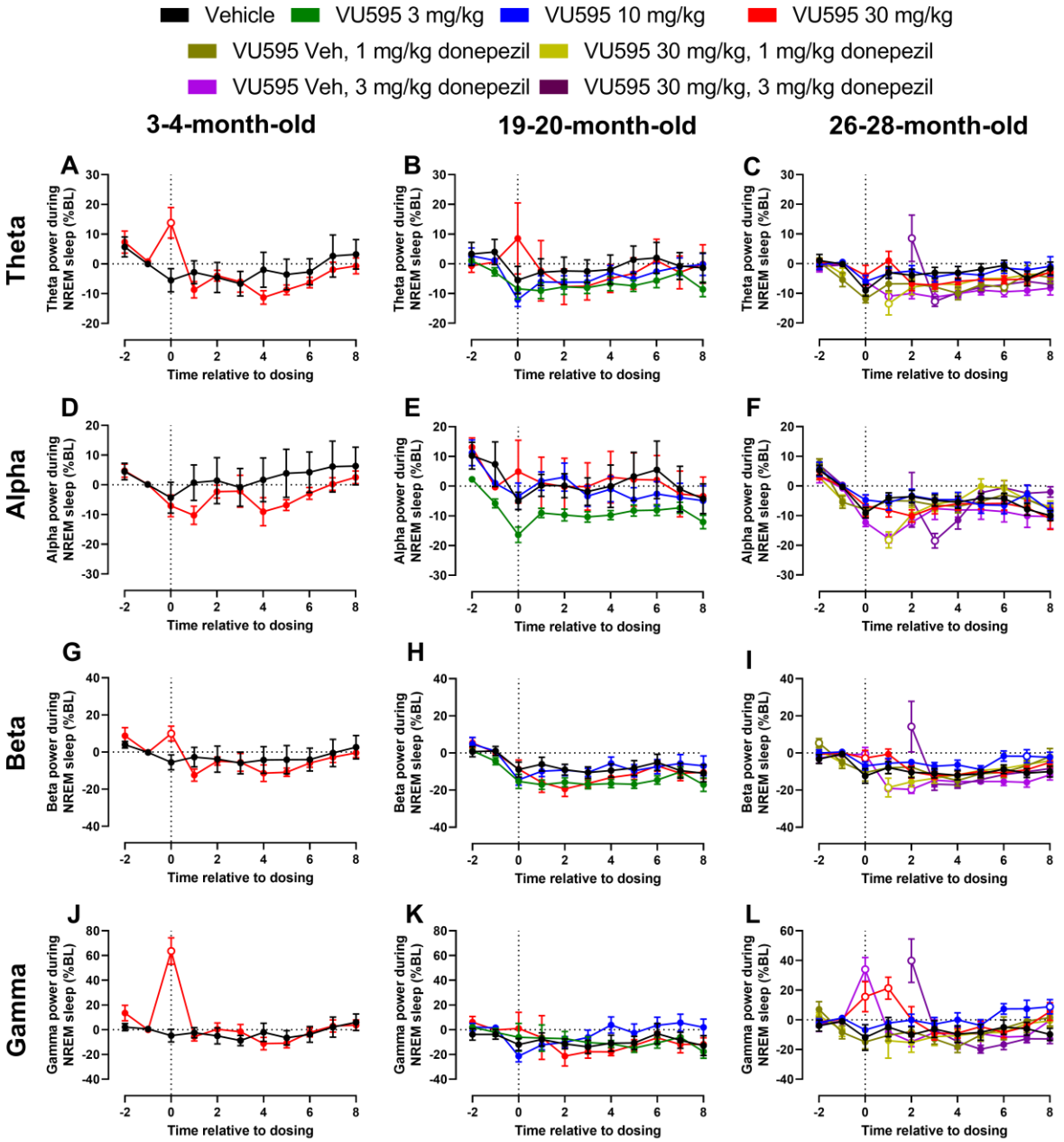

Figure 4.9. Time dependent effects of VU0453595 +/- donepezil on spectral power bands during NREM sleep in the active phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B, C), theta (D, E, F), alpha (G, H, I), and beta (J, K, L) power bands in 3-4-month-old (A, D, G, J), 19-20-month-old (B, E, H, K) and 26-28-month-old (C, F, I, L) mice during NREM sleep epochs in the active phase following VU453595 dosing at time 0. 30 mg/kg VU0453595 produced a transient increase in theta power during NREM sleep in 3-4-month-old mice (A), with no effect seen in 19-20-month-old mice (B) or 26-26-month-old mice (C). Donepezil dosed at 3 mg/kg produced decreased theta power, when 3 mg/kg donepezil was dosed in combination with 30 mg/kg transiently increased followed by decreased theta power was observed, when 1 mg/kg donepezil was dosed with 30 mg/kg VU0453595 decreased theta power was observed (C). VU0453595 dosed alone had no effect on theta power during NREM sleep in 3-4- (D), 19-20- (E) or 26-28-month-old mice (F). Donepezil alone dosed at 3 mg/kg, and in combination with 30 mg/kg VU0453595 dosed at 1 and 3 mg/kg decreased alpha power. 30 mg/kg VU0453595 produced a transient increase in beta power during NREM sleep in 3-4- (G) and 26-28-month-old mice (I), with no effect seen in 19-20-month-old mice (H). Donepezil dosed at 3 mg/kg produced decreased beta power, when 3 mg/kg donepezil was dosed in combination with 30 mg/kg transiently increased followed by decreased beta power was observed, when 1 mg/kg donepezil was dosed with 30 mg/kg VU0453595 decreased beta power was observed (I). 30 mg/kg VU0453595 increased gamma power during NREM sleep in 3-4- (J) and 26-28-month-old mice (L), with no effect observed in 19-20-month-old mice (K). 3 mg/kg donepezil alone, and in combination with 30 mg/kg VU0453595 increased gamma power during NREM sleep in 26-28-month-old mice (L). Data are expressed as means ± S.E.M. in 1-hour bins, n=7-17/group, all time points in time courses contain n=7-17 mice. Groups with fewer than 11 are due to not all mice displaying NREM sleep n=11-17 mice tested per condition, open symbols indicate p<0.05 compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 4.2 for full statistical analysis.

donepezil with 30 mg/kg VU0453595 (Figure 4.8). In the 1-2 hours following dosing during NREM sleep in 26-28-month-old mice, 30 mg/kg VU0453595 reduced delta power and increased gamma power. 1 mg/kg donepezil with 30 mg/kg VU0453595 and 3 mg/kg donepezil alone produced an increase in spectral power at 3Hz, while 1 mg/kg donepezil alone produced an increase in spectral power at 2Hz (main effect of dose, $p=0.0111$; frequency, $p<0.0001$; and dose x frequency interaction, $p<0.0001$) (Figure 4.7J). In 26-28-month-old mice, 30 mg/kg VU0453595 alone, 3 mg/kg donepezil alone and 3 mg/kg donepezil dosed with 30 mg/kg VU0453595 produced reduced delta power (SWA) during NREM sleep, while 30 mg/kg VU0453595 dosed with 1 mg/kg donepezil produced an increase in delta power (SWA) during NREM sleep (from -2 to 0hrs: no main effect of dose; main effect of time, $p<0.0001$; and dose x time interaction, $p=0.0018$ and from 1 to 8hrs: main effect of dose, $p=0.0159$, time, $p<0.0001$; and dose x time interaction, $p<0.0001$) (Figure 4.7L). Additionally, 3 mg/kg donepezil with 30 mg/kg VU0453595 increased total theta, beta and gamma, and reduced total alpha power during NREM sleep, while 3 mg/kg donepezil alone and 1 mg/kg donepezil with 30 mg/kg VU0453595 decreased total theta, alpha

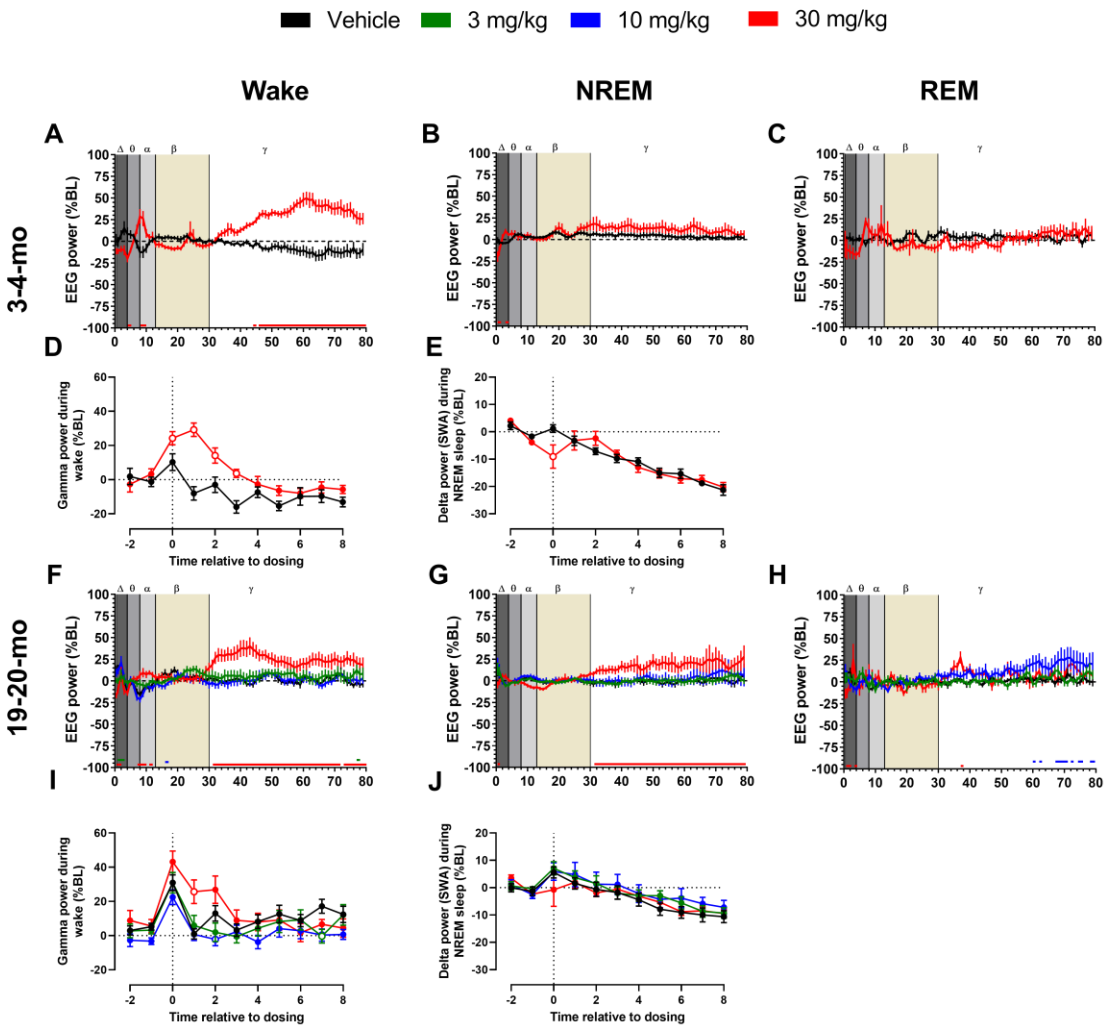

Figure 4.10. VU0453595 increased gamma power during wake, during the inactive phase in 3-4 and 19-20-month-old mice. Shown is the relative spectral power during wake (A, F), NREM sleep (B, G) and REM sleep (C, H) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline, gamma power during wake across the inactive phase (D, F) and relative delta power (SWA) during NREM sleep across the inactive phase (E, J), during the inactive phase in 3-4- (A-E) and 19-20-month-old (F-J) mice. In 3-4-month-old mice, during wake epochs 30 mg/kg VU0453595 increased alpha and gamma powers, and reduced delta powers (A) with an increased total gamma following dosing (D). VU0453595 produced decreased relative spectral power at 0.5Hz and increased relative spectral power at 3 Hz during NREM sleep in the 1-2-hrs following dosing (B), a transient decrease in delta power (SWA) during NREM sleep following dosing in the inactive phase in young mice was observed (E). In 19-20-month-old mice 30 mg/kg VU0453595 increased alpha and gamma power and reduced delta power during wake epochs (F) with transient increase in total gamma power observed following dosing (I). During NREM sleep VU0453595 reduced power at 0.5Hz and increased power in gamma frequencies (G), with no change in delta power (SWA) during NREM sleep observed (J). During REM sleep in the inactive phase in 19-20-month-old mice 30 mg/kg VU0453595 decreased relative power at 0.5-1Hz and increased relative power at 3Hz, while 10 mg/kg increased spectral power in gamma frequencies (H). Gray/tan shading represents frequency bands (∆, delta 0.5-4 Hz; θ theta 4-8 Hz; α alpha, 8-13 Hz; β beta, 13-30 Hz; γ gamma 30-80 Hz). Data are expressed as means ± S.E.M. in 1Hz bins (A, B, C, F, G, H) and means ± S.E.M. in 1-hour bins. (D, E, I, J), n=11-14/group, all time points in time courses contain n=12-14 mice (D, E, I, J). Groups with fewer than 14 are due to not all mice displaying NREM or REM sleep, n=14 mice tested per condition. Solid bars indicate $p<0.05$ compared to vehicle (A, B, C, F, G, H), open symbols indicate $p<0.05$ compared to vehicle (D, E, I, J L) (RM 2-way ANOVA or RM mixed effect model matching by both factors followed by Sidak's (A-E) or Dunnett's test (F-J)). See table 4.2 for full statistical analysis.

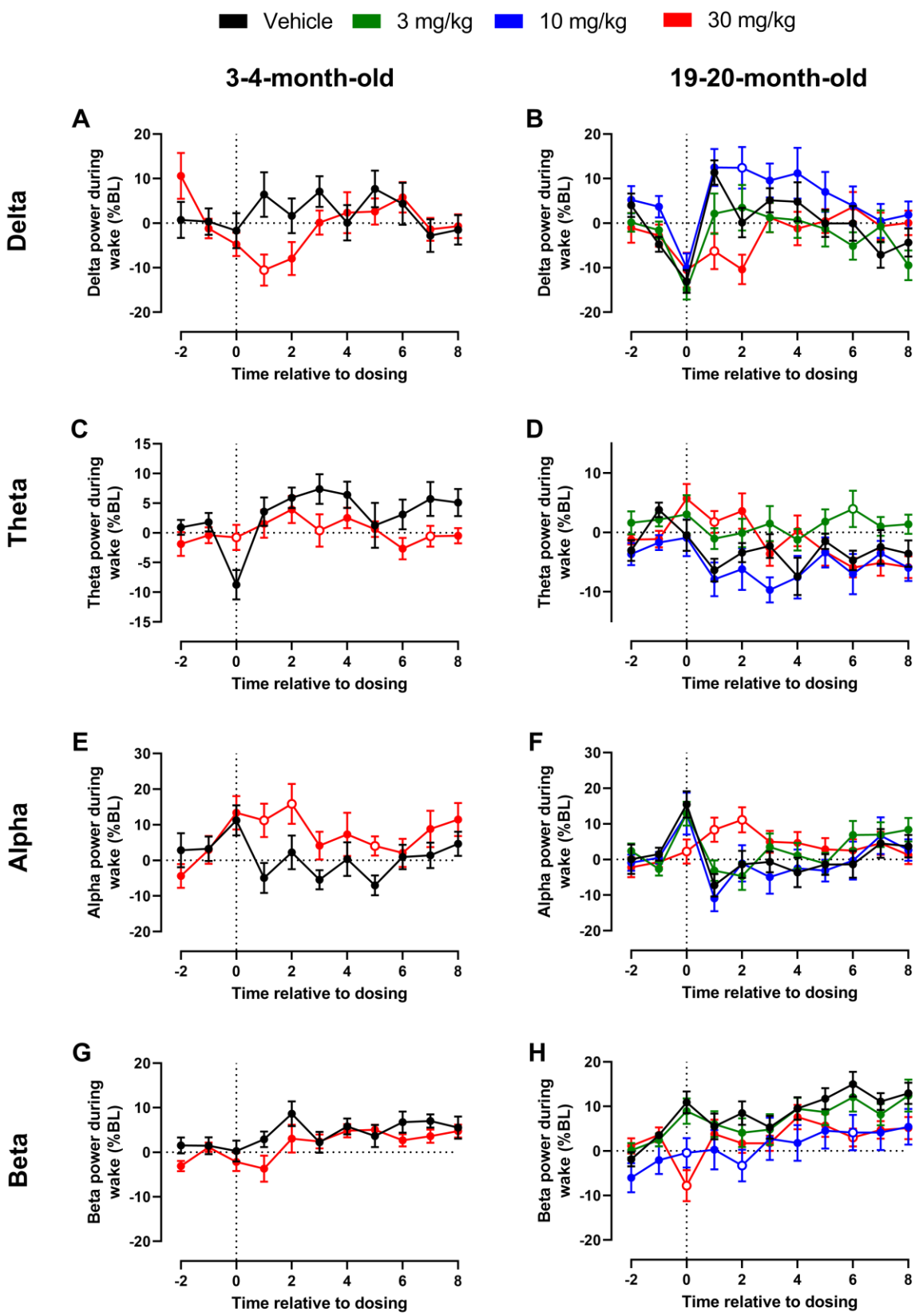
Vehicle
3 mg/kg
10 mg/kg
30 mg/kg
3-4-month-old
19-20-month-old
Delta
Theta
Alpha
Beta
A
B
C
D
E
F
G
H
Delta power during wake (%BL)
Theta power during wake (%BL)
Alpha power during wake (%BL)
Beta power during wake (%BL)
Time relative to dosing

Figure 4.11. Time dependent effects of VU0453595 on spectral power bands during wake in the inactive phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B), theta (C, D), alpha (E, F), and beta (G, H) power bands in 3-4-month-old (A, C, E, G) and 19-20-month-old (B, D, F, H) mice during wake epochs in the active phase following VU0453595 dosing at time 0. 30 mg/kg VU0453595 produced decreased delta power in 3-4- (A) and 19-20-month-old mice with a transient increase seen following 10 mg/kg dosing in 19-20-month-old mice (B). In 3-4-month-old mice fan increase and then decrease in theta power during wake was observed following 30 mg/kg VU0453595 dosing (C). In 19-20-month-old mice increased theta power was observed following 3 and 30 mg/kg VU0453595 dosing (D). Increased alpha power during wake was observed in both 3-4- (E) and 19-20-month-old mice (F). 30 mg/kg VU0453595 produced no effect on beta power in 3-4-month-old mice (G), with a significant decrease in beta power during wake observed following 10 and 30 mg/kg dosing in 19-20-month-old mice. Data are expressed as means ± S.E.M. in 1-hour bins, n=14/group, open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 4.2 for full statistical analysis.

and beta with 30 mg/kg VU0453595 alone and 3 mg/kg donepezil alone increasing gamma power (Figure 4.9).

The M_1 mAChR PAM VU0453595 increased arousal during wake epochs in the inactive phase in young and aged mice*.*

in the inactive phase in young mice, with a modest increase in alpha and reduction in theta frequencies also seen (main effect of dose $p=0.0002$; frequency, $p<0.0001$; and dose x frequency interaction, $p<0.0001$) (Figure 4.10A). Consistent with this, increased total gamma power was observed following 30 mg/kg VU0453595 dosing (main effect of dose, $p=0.0080$; time, $p<0.0001$; and dose x time interaction $p<0.0001$). Consistent with this shift to higher powers, reductions in total delta and theta power are observed, with increased alpha power (Figure 4.11). During NREM sleep 30 mg/kg VU0453595 decreased 0.5Hz powers and increased 3Hz powers in the 1-2 hours following dosing (no main effect of dose; main effect of frequency, $p<0.0001$; and dose x frequency interaction, $p<0.0001$) (Figure 4.10B). When assessing total delta power (SWA) during NREM sleep after dosing a transient reduction in delta power was observed (no main effect of dose; main effect of time $p<0.0001$; and dose x time interaction, $p=0.0008$) (Figure 4.10E). Additionally, transiently increased theta, beta and gamma powers are observed during NREM sleep following 30 mg/kg VU0453595 dosing (Figure 4.12). During REM sleep no effect at any frequency following posthoc tests following dosing with 30 mg/kg VU0453595 is observed (no

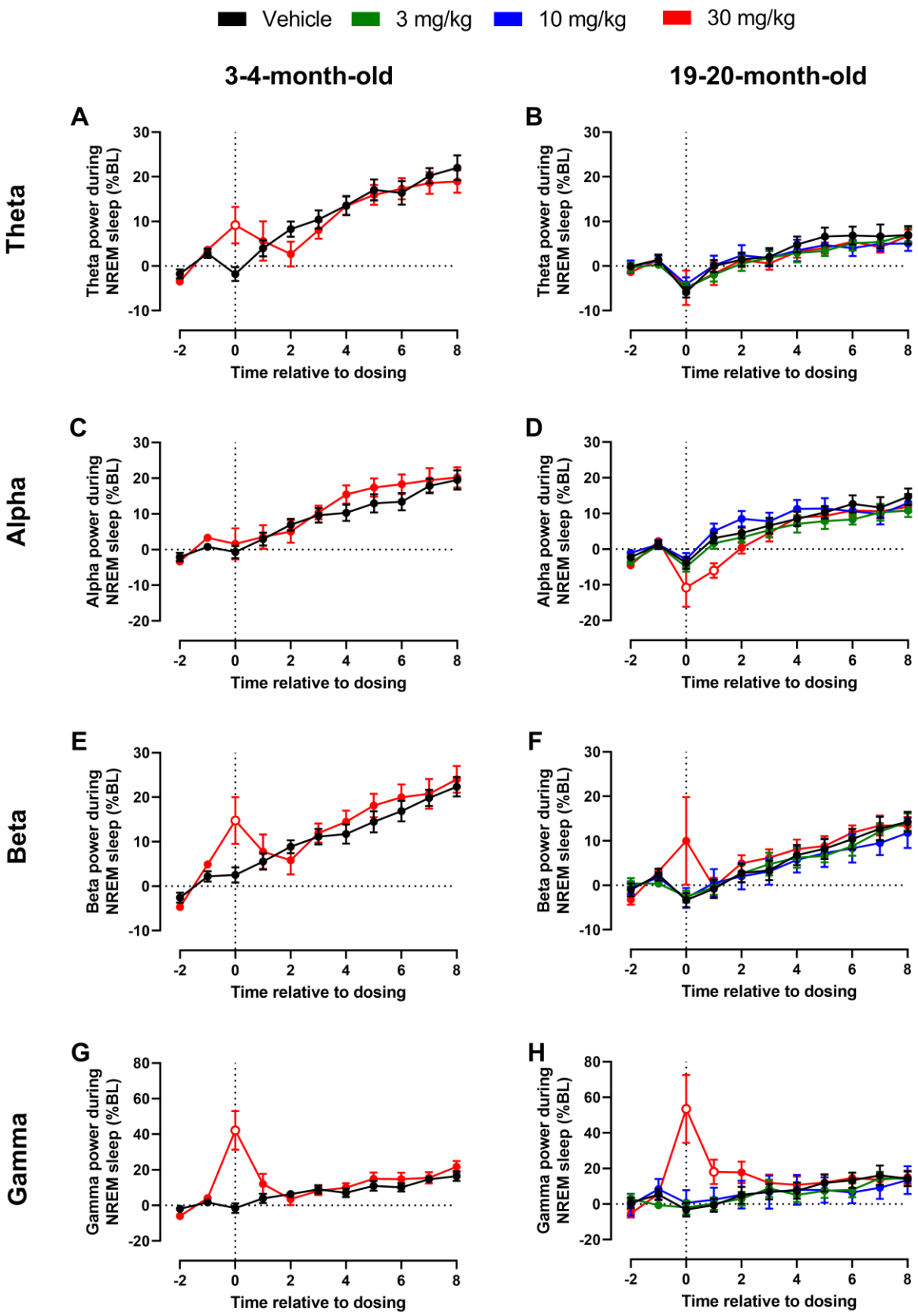
Vehicle
3 mg/kg
10 mg/kg
30 mg/kg
3-4-month-old
19-20-month-old
Theta
Alpha
Beta
Gamma
A
B
C
D
E
F
G
H
Theta power during NREM sleep (%BL)
Alpha power during NREM sleep (%BL)
Beta power during NREM sleep (%BL)
Gamma power during NREM sleep (%BL)
Time relative to dosing

Figure 4.12. Time dependent effects of VU0453595 on spectral power bands during NREM sleep in the inactive phase. Shown is the power relative the 1–2-hour baseline following light change within delta (A, B), theta (C, D), alpha (E, F), and beta (G, H) power bands in 3-4-month-old (A, C, E, G) and 19-20-month-old (B, D, F, H) mice during NREM sleep epochs in the active phase following VU0453595 dosing at time 0. 30 mg/kg VU0453595 increased theta power during NREM sleep in 3-4-month-old mice (A), with no effect observed in 19-20-month-old mice (B). VU0453595 produced no effect on alpha power in 3-4-month-old mice (C), with a decreased alpha power observed following 30 mg/kg VU0453595 dosing in 19-20-month-old mice (D). 30 mg/kg VU0453595 increased beta power in 3-4-month-old mice (E), with no effect observed in 19-20-month-old mice. 30 mg/kg VU0453595 increased gamma power during NREM sleep in 3-4- (G) and 19-20-month-old mice (H). Data are expressed as means ± S.E.M. in 1-hour bins, all timepoints contain n= 12-14 mice, n=14/group tested, missing data due to mice not entering NREM sleep during specific timepoint. Open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 4.2 for full statistical analysis.

main effect dose; main effect of frequency, p=0.0207; and dose x frequency, p=0.0019) (Figure 4.10C).

VU0453595 30 mg/kg increased relative power across gamma frequencies when dosed VU0453595 30 mg/kg increased relative power across gamma frequencies when dosed in the inactive phase in 19-20-month-old mice (main effect of dose, p=0.0036; frequency, p<0.0001; and dose x frequency interaction, p<0.0001) (Figure 4.10F). Consistent with these findings increased total gamma was seen following 30 mg/kg VU0453595 (no main effect of dose; main effect of time, p<0.0001; and dose x time interaction, p<0.0001) (Figure 4.10I). 30 mg/kg VU0453595 also produced decreased delta and beta, and increased theta and alpha during wake epochs (Figure 4.11). During NREM sleep VU0453595 produced a significant increase in gamma frequency power and a reduction in spectral power at 0.5Hz (no main effect of dose; main effect of frequency, p<0.0001; and dose x frequency interaction, p<0.0001). No effect is observed when assessing total NREM sleep delta power (SWA) following dosing with VU0453595 (no main effect of dose; main effect of time, p<0.0001; and no dose x time interaction) (Figure 4.10J). In 30 mg/kg VU0453595 produced decreased alpha power and increased gamma power during NREM sleep epochs (Figure 4.12). During REM sleep epochs 30 mg/kg VU0453595 produced decreased spectral power at 0.5-1Hz and increased spectral power at 3Hz, while 10 mg/kg produced increased spectral power in gamma frequencies (no main effect of dose; main effect of time, p<0.0001; and dose x time interaction, p>0.0001) (Figure 4.10H).

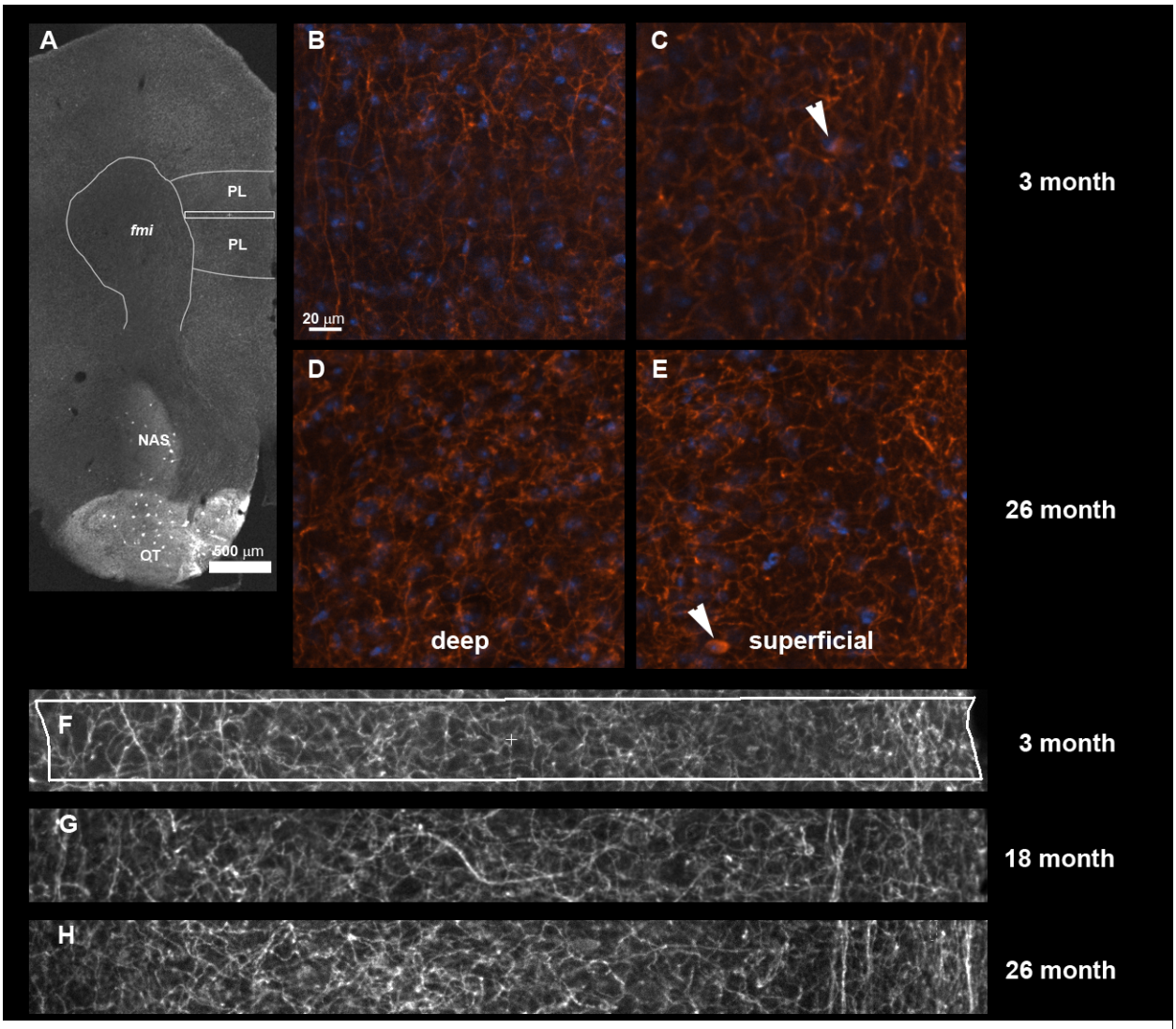

Figure 4.13. Innervation of the prefrontal cortex of young and non-pathologically aged C57BL/6 mice as revealed by choline acetyltransferase (ChAT)-like immunoreactivity (-li). In a coronal section through the PFC, the 50-um-wide strip indicates the location where ChAT-li fibers were traced and quantitated. Note the presence of ChAT-li interneurons in the nucleus accumbens and olfactory tubercle (A). Red immunolabeling represents the dense ChAT-li fiber network that is seen in the deep (B and D) and superficial layers (C and E) layers of the PFC of young (B-C) and aged (D-E) mice. In between these fibers blue DAPI staining delineated neuronal nuclei. The arrowheads in (C) and (E) point to ChAT-li interneurons occasionally encountered in the superficial layers. Photomicrographs in F-H illustrate the ChAT-li fiber innervation (F-I) in a 50-um wide strip of the PFC of young (F) and aged (18 month-old [G] or 26-month-old [H]) mice. In (I) the tracing of ChAT-li fibers shown in (F) is illustrated where green and yellow lines depict fibers detected by autotracing using Nikon Elements GA3 analysis and manual tracing, respectively. scale bar in B also applies to C-E and scale bar in I applies also to F-H. fmi, forceps minor of the corpus callosum; NAS, nucleus accumbens; OT, olfactory tubercle; PL, prelimbic area.

No age-related effect on ChAT-positive fiber density is observed in the mouse prefrontal cortex.

To assess if the age-related differences in VU0453595 efficacy can be attributed to age-related changes in the cholinergic innervation, we determined the cholinergic fiber density in the PFC, specifically in the prelimbic area of the medial PFC. Representative images are seen in Figure 14.3. Additionally, we measured the cortical thickness of the PFC. Irrespective of whether

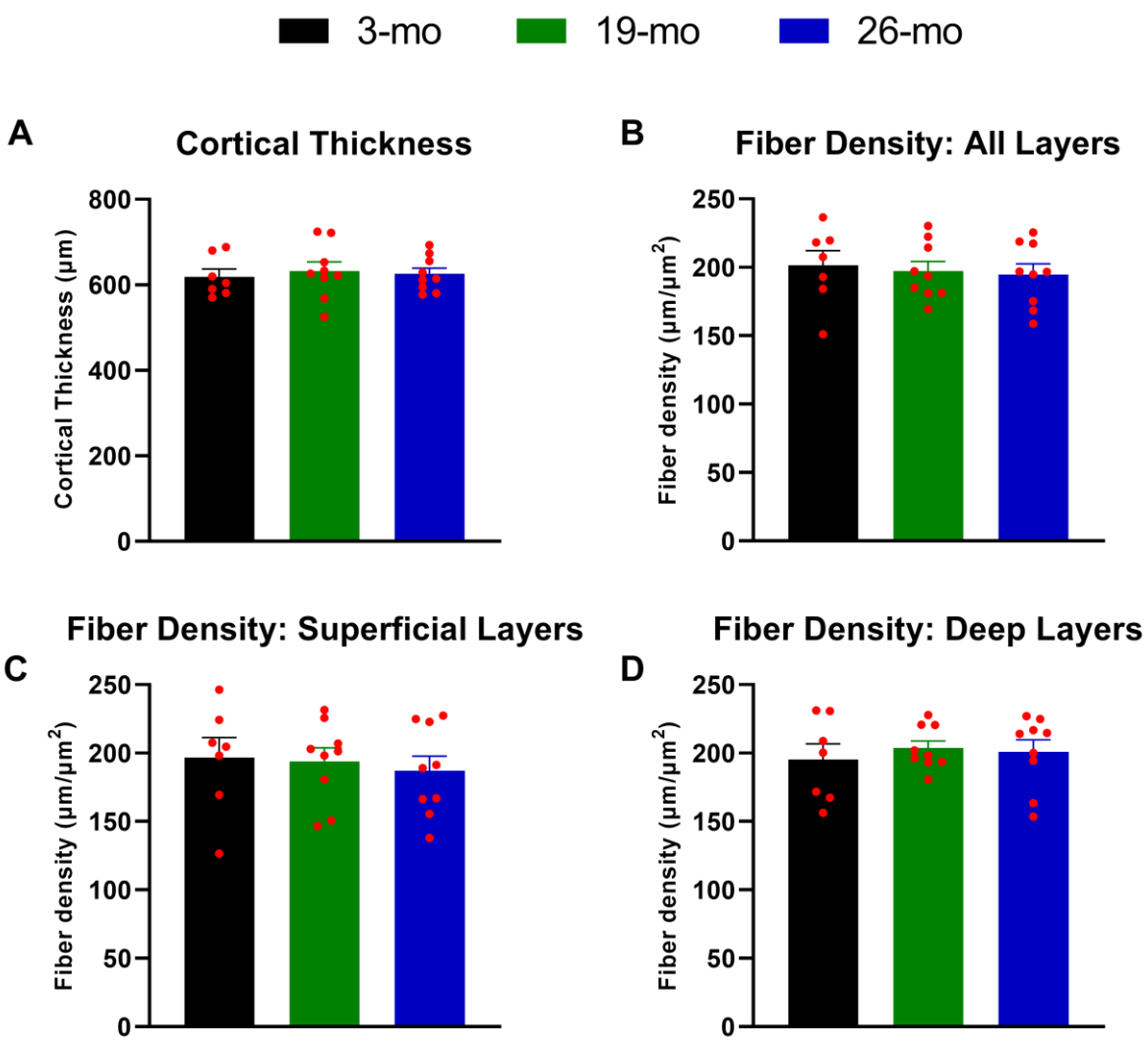

Figure 4.14. 3-, 19- and 26-month-old mice showed no difference in cholinergic fiber density or cortical thickness in the prefrontal cortex. Shown is the cortical thickness (A), ChAT positive fiber density across all layers (B) and in the superficial, I-III, layers (C) and in the deep layers, V-VI, in the (D) in the prefrontal cortex in 3-, 19- and 26-month-old mice. No age-related effect on cortical thickness (A), total ChAT positive fiber density (B), ChAT positive fiber density in the superficial layers (C) or ChAT positive fiber density in the deep layers (D). Data are expressed as overall means ± S.E.M., n=7-9/group. See table 4.2 for full statistical analysis.

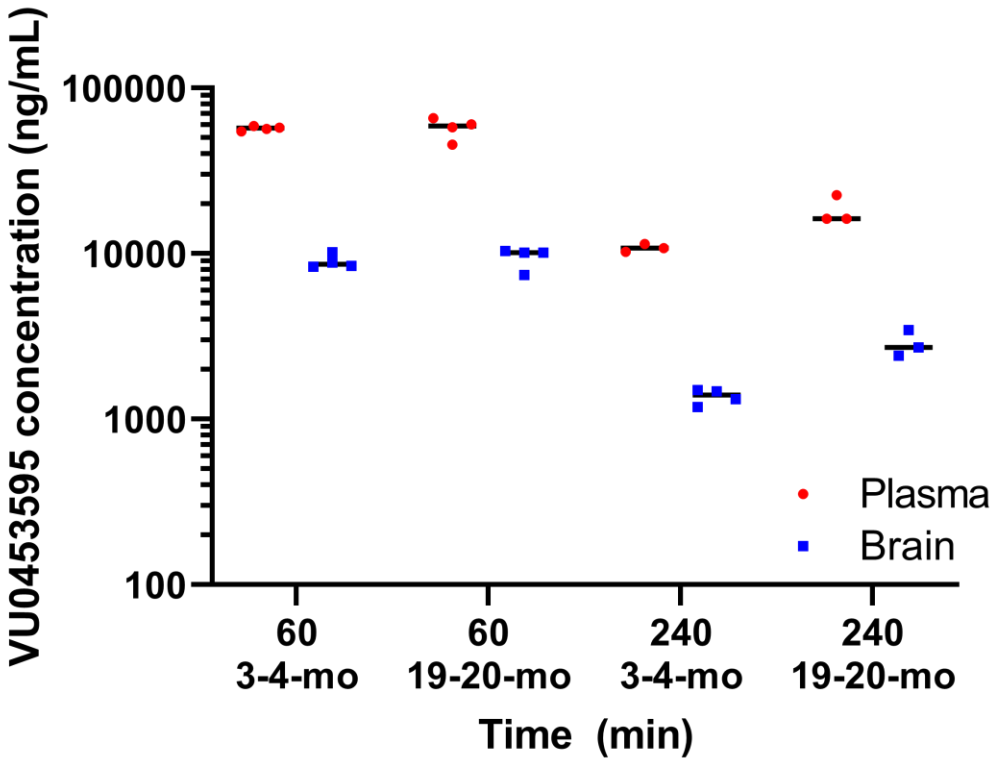

Figure 4.15. VU0453595 displays no pK difference based on age. Shown is the concentration of VU0453595 in plasma and brain in 3-4- and 19-20-month-old mice 60- and 240-minutes after IP dosing.

ChAT-li fiber density was assessed in the entire PFC (layers I-VI), or separately in the superficial (I-III) or deep (V-VI) layers of the PFC, no age-related changes were observed (Figure 4.14B-D). Furthermore, no age-related change in PFC thickness was seen (Figure 4.14A).

VU0453595 pharmacokinetics does not vary in 3-4- and 19-20-month-old mice, and VU0453595 does not produce side effects in 19-20-month-old display.

3-4- and 19-20-month-old mice were dosed with 30 mg/kg VU0453595 with blood and brain samples collected non-serially at 1 and 4 hours. Plasma and brain exposures were comparable in 3-4- and 19-20-month-old mice at 1 and 4 hours post VU0453595 administration (Figure 4.15). K_p in 3-4-month-old mice 1 hour post dosing was calculated to be 0.16, and in 19-20-month-old mice was calculated to be 0.17.

In 19-20-month-old mice tested during the inactive and active phase, 30 mg/kg VU0453595 produced no increase in cholinergic-mediated adverse effects on the modified Irwin test battery when compared to vehicle treated mice (Table 4.1).

4.4. Discussion

In non-pathological aging and AD, a wide array of sleep-wake architecture deficits is seen, which may contribute to cognitive deficits seen in aging and AD (Bubu et al., 2017; Lim et al., 2013; Mander et al., 2017). Furthermore, a bidirectional relationship between sleep disturbances and AD pathology is seen, with amyloid and tau pathology creating greater sleep disturbances and sleep disturbances increasing AD pathology (Ju et al., 2017; C. Wang and Holtzman, 2020). The current findings have indicated that the effects of the M_1 mAChR PAM, VU0453595, are dependent on the age of the mice at the time of dosing, and the circadian time point when dosing is performed.

VU0453595 promoted wakefulness in the active phase in 19-20- and 26-28-month-old mice but not 3-4-month-old mice, where increased wakefulness was only observed in the inactive phase. Young rodents have been previously demonstrated to display circadian fluctuations in cholinergic signaling, with high levels during the active phase and low levels during the inactive phase. This circadian fluctuation disappears with aging (Mitsushima et al., 1996). This suggests that in young mice (3-4-month-old), there is a signal window to see enhancement during the inactive phase, but not the active phase where cholinergic signaling is optimal. In non-pathological aging (19-20- and 26-28-month-old), where previous data suggests central cholinergic structure is reduced in hippocampal and parietal areas (Fischer et al., 1992; Xie et al., 2019) and normal circadian fluctuations in cholinergic signaling are lost, cholinergic signaling is presumably sub-optimal across the circadian rhythm. With this sub-optimal cholinergic signaling the M_1 mAChR PAM VU0453595 can enhance wakefulness during the active and inactive phases.

The M_1 mAChR PAM VU0453595 boosted gamma power, a recognized correlate of arousal (Buzsáki and Silva, 2012), during the active phase in 19-20-month-old mice. However, limited effects on arousal were observed in 3-4- mice or 26-28-month-old mice during the active phase. In 3-4-month-old mice, the reduced effect on arousal is likely due to cholinergic signaling already being optimal, similar to previously hypothesized for the lack of VU0453595-dependent effect on wakefulness. With increasing age, central cholinergic integrity declines, and age-related reductions in arousal are observed (Russell et al., 2023). As such, in 19-20-month-old mice, the M_1 mAChR PAM VU0453595 increased arousal in both the active and inactive phases, normalizing active phase deficits. With further increased age, in 26-28-month-old mice, the enhancement of arousal following VU0453595 dosing during the active declined. This reduction in effect size at an advanced age is consistent with our previously published inactive phase data (Gould et al., 2020). We hypothesize that this is due to a further age-related decline in cholinergic signaling, with the efficacy of PAMs being dependent on levels of the endogenous ligand (Bubser et al., 2012). To test whether this lack of efficacy was due to declined cholinergic signaling, we dosed 30 mg/kg VU0453595 in combination with 1 and 3 mg/kg of donepezil. These doses produced no effect on arousal when dosed alone. 3 mg/kg donepezil dosed in conjunction with 30 mg/kg VU0453595 produced robust increases in gamma power, suggesting that boosting cholinergic signaling in these aged mice is sufficient to restore the effects of the M_1 mAChR PAM VU0453595.

In trying to understand the effects of an M_1 mAChR PAM at advanced age it is important to consider changes in central cholinergic integrity may impact observed efficacy. To further investigate the reduced effects of VU0453595 on arousal with increasing age we assessed the integrity of cholinergic terminals in the PFC. The PFC is richly innervated by projections from the cholinergic basal forebrain (M. M. Mesulam, 1990), an area which degenerates in non-pathological aging and AD (Bartus et al., 1982; Fischer et al., 1992) and is vitally important in normal wake and arousal (B. E. Jones, 2020). Interestingly we observed no decrease in

cholinergic fiber density within the PFC. Previous data using similar methods in 25-month-old mice identified reduced cholinergic fiber density in the hippocampal and parietal cortex (Xie et al., 2019). Within the hippocampus, cholinergic signaling is known to modulate theta frequency oscillations (Gu and Yakel, 2022; X. Ma et al., 2020), which couple to PFC gamma oscillations (Tamura et al., 2017). It is possible VU0453595 induced wake promotion is primarily modulated through cortical cholinergic basal forebrain projections, which are known to be important for wakefulness (B. E. Jones, 2020). While VU0453595-dependent increases in arousal may be through cholinergic modulation of hippocampal theta oscillations coupling to gamma oscillations. A decline in hippocampal cholinergic projections with relatively preserved PFC cholinergic structure may explain why the wake-promoting effects of VU0453595 are preserved in 26-28-month-old mice, and in our previous data in 22-26-month-old mice (Gould et al., 2020), while effects on arousal are reduced in advanced age. However, the currently observed lack of age-related change in cholinergic fiber density within the PFC does not definitively answer whether there are changes in cholinergic neuron function, or the micro-structure of cholinergic neurons within the PFC. Previous studies in rodents have suggested age-related decreases in cholinergic boutons within the PFC (Casu et al., 2002), while other studies have suggested age-related differences in the circadian-dependent release of ACh in the PFC (Mitsushima et al., 1996). In addition, the reduced VU0453595-dependent effects observed on arousal could be due to reductions in M_1 mAChR. Future studies will investigate M_1 mAChR levels throughout cortical and limbic regions in non-pathological aging.

In conclusion, these data suggest that in non-pathologically aged mice, at an age where reduced cholinergic fiber density has been previously demonstrated (Xie et al., 2019), the M_1 mAChR PAM VU0453595 can enhance wakefulness and arousal in both the inactive and active phases in aged mice, either alone or in combination with donepezil in 26-28-month-old mice. Importantly, VU0453595 produced no significant adverse effects on the Modified Irwin Test Battery at 30 mg/kg. These findings are crucial as they indicate that the M_1 mAChR PAM

VU0453595 can enhance wakefulness and arousal during the active period in mice with a cholinergic deficit at doses that do not produce cholinergic side effects, suggesting that M_1 mAChR PAMs could have efficacy in enhancing wake and arousal in AD clinical populations with dosing during the daytime. This supports the further development of M_1 mAChR PAMs for the symptomatic treatment of AD and suggests that M_1 mAChR PAMs may be AChEI-sparing in individuals with increased cholinergic loss.

	ACTIVE								INACTIVE							
	Vehicle				30 mg/kg VU0453595				Vehicle				30 mg/kg VU0453595			
Time (minutes)	30	60	120	240	30	60	120	240	30	60	120	240	30	60	120	240
Autonomic Nervous System																
Ptosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exophtalmus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corneal reflex loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pinna reflex loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Respiratory rate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Writing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tail erection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lacrimation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vasodilation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Skin color	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Irritability	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baseline pupil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pupil reaction	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Somatomotor Systems																
Motor activity	0.167	0.167	0.167	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arch/Roll	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0	0	0	0	0	0	0	0	0.167	0	0	0
Leg weakness	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rigid stance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spraddle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placing loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Grasping loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Righting loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Catalepsy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tail pinch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	**0.167**	**0.167**	**0.167**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.167**	**0.000**	**0.000**	**0.000**
	Dose $F_{(1, 10)}$=0.0400, p=0.8455; Time $F_{(3, 33)}$=0.5576, p=0.6467								Dose $F_{(1, 10)}$=1.000, p=0.3409; Time $F_{(3, 33)}$=1.000, p=0.4051							
	For all behaviors scored: 0 = normal, 1 = mild effect and 2 = severe effect															

Table 4.1 VU0453595 does not produce cholinergic adverse effects on modified Irwin in nonpathologically aged mice.

Figure	Age	Experiment	Measure	Phase	Statistical Test	Comparison	Degrees of freedom	F or t	P	*	Group Size	Post hoc results
1a	3-4mo	VU0453595 effects on time in wake	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	19.89	0.0008	***	N=13	None
						Time	11, 132	76.73	<0.0001	****		
						Dose x Time	11, 132	0.3593	0.9692	ns		
1b	3-4mo	VU0453595 effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	19.85	0.0008	***	N=13	None
						Time	11, 132	68.52	<0.0001	****		
						Dose x Time	11, 132	0.3176	0.981	ns		
1c	3-4mo	VU0453595 effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	0.4997	0.4931	ns	N=13	30 mg/kg vs Veh time: ZT2 and 6
						Time	11, 132	86.56	<0.0001	****		
						Dose x Time	11, 132	2.639	0.0044	**		
1d	3-4mo	VU0453595 effects on time in wake	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	19.89	0.0008	***	N=13	30 mg/kg vs Veh time: ZT12-24, ZT0-12
						Time	1, 12	460.3	<0.0001	****		
						Dose x Time	1, 12	0.03698	0.8454	ns		
1e	3-4mo	VU0453595 effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	19.85	0.0008	***	N=13	30 mg/kg vs Veh time: ZT12-24
						Time	1, 12	415.7	<0.0001	****		
						Dose x Time	1, 12	1.103	0.3144	ns		
1f	3-4mo	VU0453595 effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	0.4997	0.4931	ns	N=13	None
						Time	1, 12	723.4	<0.0001	****		
						Dose x Time	1, 12	7.075	0.0208	*		
1g	19-20mo	VU0453595 effects on time in wake	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.793	0.0062	**	N=14	10 mg/kg vs Veh time: ZT18 30 mg/kg vs Veh time: ZT14, 20 and 22
						Time	11, 143	66.55	<0.0001	****		
						Dose x Time	33, 429	2.057	0.0007	***		
1h	19-20mo	VU0453595 effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	5.856	0.0021	**	N=14	10 mg/kg vs Veh time: ZT18 30 mg/kg vs Veh time: ZT14, 20 and 22
						Time	11, 143	60.13	<0.0001	****		
						Dose x Time	33, 429	2.151	0.0003	***		
1i	19-20mo	VU0453595 effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	0.6581	0.5828	ns	N=14	N/A
						Time	11, 143	86.57	<0.0001	****		
						Dose x Time	33, 429	0.9868	0.4914	ns		
1j	19-20mo	VU0453595 effects on time in wake	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.793	0.0062	**	N=14	10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24
						Time	1, 13	625.0	<0.0001	****		
						Dose x Time	3, 39	6.527	0.0011	**		
1k	19-20mo	VU0453595 effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	5.856	0.0021	**	N=14	10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24
						Time	1, 13	592.7	<0.0001	****		
						Dose x Time	3, 39	6.682	0.0010	***		
1l	19-20mo	VU0453595 effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	0.6581	0.5828	ns	N=14	N/A
						Time	1, 13	497.1	<0.0001	****		
						Dose x Time	3, 39	1.683	0.1865	ns		
1m	25-27mo	VU0453595 +/- Donepezil effects on time in wake	Duration (min/2hr)	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 69	8.058	<0.0001	****	N=11-17	30 mg/kg VU0453595 vs Veh: ZT14 30 mg/kg VU0453595 + Donepezil 1mg/kg vs Veh: ZT14 and 16 30 mg/kg VU0453595 + Donepezil 3 mg/kg vs Veh: ZT14 , 16 and 0
						Time	11, 176	164.1	<0.0001	****		
						Dose x Time	66, 768	3.464	<0.0001	****		
1n	25-27mo	VU0453595 +/- Donepezil effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 69	7.406	<0.0001	****	N=11-17	30 mg/kg VU0453595 vs Veh: ZT14 30 mg/kg VU0453595 + 1mg/kg Donepezil vs Veh: ZT14 and 16 30 mg/kg VU0453595 + 3 mg/kg Donepezil vs Veh: ZT14 , 16 and 0 3 mg/kg Donepezil vs Veh:ZT14
						Time	11, 176	132.0	<0.0001	****		
						Dose x Time	66, 768	3.390	<0.0001	****		
1o	25-27mo	VU0453595 +/- Donepezil effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 69	0.1735	0.9834	ns	N=11-17	30 mg/kg VU0453595 + 3 mg/kg Donepezil vs Veh: ZT20
						Time	11, 176	239.0	<0.0001	****		
						Dose x Time	66, 768	2.334	<0.0001	****		
1p	25-27mo	VU0453595 +/- Donepezil effects on time in wake	Duration (min/12hr)	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 176	6.781	<0.0001	****	N=11-17	30 mg/kg VU0453595 + 1mg/kg Donepezil vs Veh: ZT12-24 30 mg/kg VU0453595 + 3 mg/kg Donepezil vs Veh: ZT12-24
						Time	1, 176	1657	<0.0001	****		
						Dose x Time	6, 176	3.173	0.0056	**		
1q	25-27mo	VU0453595 +/- Donepezil effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 176	6.722	<0.0001	****	N=11-17	30 mg/kg VU0453595 + 1mg/kg Donepezil vs Veh: ZT12-24 30 mg/kg VU0453595 + 3 mg/kg Donepezil vs Veh: ZT12-24
						Time	1, 176	1208	<0.0001	****		
						Dose x Time	6, 176	2.366	0.0319	*		
1r	25-27mo	VU0453595 +/- Donepezil effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 176	0.6412	0.6970	ns	N=11-17	N/A
						Time	1, 176	1053	<0.0001	****		
						Dose x Time	6, 176	1.217	0.3141	ns		

2a	3-4mo	VU0453595 effects on Wake Bout #	Direct comparison	Active	T-test	Dose	12	0.4634	0.6514	ns	N=14	N/A
2b	3-4mo	VU0453595 effects on Wake Bout length	Direct comparison	Active	T-test	Dose	12	0.4146	0.6857	ns	N=14	N/A
2c	19-20mo	VU0453595 effects on Wake Bout #	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	1.005	0.4009	ns	N=14	N/A
2d	19-20mo	VU0453595 effects on Wake Bout length	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	2.006	0.1291	ns	N=14	N/A
2e	25-27mo	VU0453595 +/- Donepezil effects on Wake Bout #	Direct comparison	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 72	1.902	0.0921	ns	N=11-17	N/A
2f	25-27mo	VU0453595 +/- Donepezil effects on Wake Bout length	Direct comparison	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 88	3.861	0.0018	**	N=11-17	30 mg/kg VU0453595 +3 mg/kg Donepezil vs Veh: p=0.0004
3a	3-4mo	VU0453595 effects on NREM Bout #	Direct comparison	Active	T-test	Dose	12	0.1381	0.8924	ns	N=13	N/A
3b	3-4mo	VU0453595 effects on NREM Bout length	Direct comparison	Active	T-test	Dose	12	0.6997	0.4974	ns	N=13	N/A
3c	19-20mo	VU0453595 effects on NREM Bout #	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	1.340	0.2754	ns	N=14	N/A
3d	19-20mo	VU0453595 effects on NREM Bout length	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	0.9727	0.4154	ns	N=14	N/A
3e	25-27mo	VU0453595 +/- Donepezil effects on NREM Bout #	Direct comparison	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 72	1.890	0.0942	ns	N=11-17	N/A
3f	25-27mo	VU0453595 +/- Donepezil effects on NREM Bout length	Direct comparison	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 71	2.981	0.0118	*	N=11-17	3 mg/kg Donepezil vs Veh: p=0.0008
4a	3-4mo	VU0453595 effects on time in wake	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	1.926	0.1885	ns	N=14	30 mg/kg vs Veh time: ZT2
						Time	11, 143	77.56	<0.0001	****		
						Dose x Time	11, 143	5.213	<0.0001	****		
4b	3-4mo	VU0453595 effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	3.446	0.0862	ns	N=14	30 mg/kg vs Veh time: ZT2
						Time	11, 143	68.19	<0.0001	****		
						Dose x Time	11, 143	4.425	<0.0001	****		
4c	3-4mo	VU0453595 effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	0.5307	0.4792	ns	N=14	30 mg/kg vs Veh time: ZT2 and 8
						Time	11, 143	82.67	<0.0001	****		
						Dose x Time	11, 143	5.395	<0.0001	****		
4d	3-4mo	VU0453595 effects on time in wake	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	1.926	0.1885	ns	N=14	30 mg/kg vs Veh time: ZT0-ZT12
						Time	1, 13	227.9	<0.0001	****		
						Dose x Time	1, 13	5.267	0.0390	*		
4e	3-4mo	VU0453595 effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	3.446	0.0862	ns	N=14	30 mg/kg vs Veh time: ZT0-ZT12
						Time	1, 13	206.1	<0.0001	****		
						Dose x Time	1, 13	6.826	0.0215	*		
4f	3-4mo	VU0453595 effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	0.5307	0.4792	ns	N=14	N/A
						Time	1, 13	323.7	<0.0001	****		
						Dose x Time	1, 13	1.960	0.1849	ns		

4g	19-20mo	VU0453595 effects on time in wake	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.628	0.0073	**	N=14	1mg/kg vs Veh time: ZT16 and 22 30 mg/kg vs Veh time: ZT2 and 4
						Time	11, 143	68.31	<0.0001	****		
						Dose x Time	33, 429	3.34	<0.0001	****		
4h	19-20mo	VU0453595 effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.81	0.0173	*	N=14	1mg/kg vs Veh time: ZT16 and 22 30 mg/kg vs Veh time: ZT2 and 4
						Time	11, 143	60.68	<0.0001	****		
						Dose x Time	33, 429	3.005	<0.0001	****		
4i	19-20mo	VU0453595 effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.448	0.2437	ns	N=14	30 mg/kg vs Veh time: ZT2, 4 and 6
						Time	11, 143	81.83	<0.0001	****		
						Dose x Time	33, 429	3.835	<0.0001	****		
4j	19-20mo	VU0453595 effects on time in wake	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.628	0.0073	**	N=14	3 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT0-12
						Time	1, 13	768.4	<0.0001	****		
						Dose x Time	3, 39	4.595	0.0076	**		
4k	19-20mo	VU0453595 effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.810	0.0173	*	N=14	3 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT0-12
						Time	1, 13	633.8	<0.0001	****		
						Dose x Time	3, 39	5.037	0.0048	**		
4l	19-20mo	VU0453595 effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.448	0.2437	ns	N=14	N/A
						Time	1, 13	297.3	<0.0001	****		
						Dose x Time	3, 39	1.626	0.1990	ns		
5a	3-4mo	VU0453595 effects on Wake Bout #	Direct comparison	Inactive	T-test	Dose	13	1.539	0.1478	ns	N=14	N/A
5b	3-4mo	VU0453595 effects on Wake Bout length	Direct comparison	Inactive	T-test	Dose	13	0.9159	0.3764	ns	N=14	N/A
5c	19-20mo	VU0453595 effects on Wake Bout #	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	3.232	0.0325	*	N=14	30 mg/kg vs Veh: p=0.0155
5d	19-20mo	VU0453595 effects on Wake Bout length	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	1.635	0.1970	ns	N=14	N/A
6a	3-4mo	VU0453595 effects on NREM Bout #	Direct comparison	Inactive	T-test	Dose	13	0.7326	0.4768	ns	N=14	N/A
6b	3-4mo	VU0453595 effects on NREM Bout length	Direct comparison	Inactive	T-test	Dose	13	2.194	0.0470	*	N=14	N/A
6c	19-20mo	VU0453595 effects on NREM Bout #	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	3.243	0.0321	*	N=14	30 mg/kg vs Veh: p=0.0162
6d	19-20mo	VU0453595 effects on NREM Bout length	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	4.080	0.0130	*	N=14	30 mg/kg vs Veh: p=0.0039
7a	3-4mo	VU0453595 effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	4.656	0.0519	ns	N=13	30 mg/kg vs Veh Freq: 56-62 and 68-71Hz
						Frequency	79, 948	14.07	<0.0001	****		
						Dose x Frequency	79, 948	5.142	<0.0001	****		
7b	3-4mo	VU0453595 effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 12	0.8184	0.3835	ns	N=12-13	None
						Frequency	79, 948	8.174	<0.0001	****		
						Dose x Frequency	79, 868	1.779	<0.0001	****		
7c	3-4mo	VU0453595 effects on gamma power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	0.3959	0.4377	ns	N=13	30 mg/kg vs Veh Time: 0hr
						Time	10, 120	17.04	<0.0001	****		
						Dose x Time	12, 120	4.018	0.0001	***		
7d	3-4mo	VU0453595 effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 12	0.2459	0.6289	ns	N=12-13	30 mg/kg vs Veh Time: 0hr
						Time	10, 120	19.13	<0.0001	****		
						Dose x Time	10, 96	4.305	<0.0001	****		
7e	19-20mo	VU0453595 effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	10.34	<0.0001	****	N=14	3 mg/kg vs Veh Freq: 2-4 and 9Hz 10 mg/kg vs Veh Freq: 3Hz 30 mg/kg vs Veh Freq: 3-4, 9, 13-18, 20-27, 33-55 and 60-79Hz
						Frequency	79, 1027	3.946	<0.0001	****		
						Dose x Frequency	237, 3081	7.615	<0.0001	****		
7f	19-20mo	VU0453595 effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.05834	0.9812	ns	N=8-14	N/A
						Frequency	79, 1027	3.882	<0.0001	****		
						Dose x Frequency	237, 2361	0.8141	0.9798	ns		
7g	19-20mo	VU0453595 effects on gamma power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.768	0.0063	**	N=14	10 mg/kg vs Veh Time: 0hr 30 mg/kg vs Veh Time: 0, 1, 2, 3 and 4hr
						Time	10, 130	18.53	<0.0001	****		
						Dose x Time	30, 390	4.853	<0.0001	****		

7h	19-20mo	VU0453595 effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.5775	0.7146	ns	N=12-14	N/A
						Time	10, 130	11.08	<0.0001	****		
						Dose x Time	30, 320	1.491	0.0512	ns		
7i	25-27mo	VU0453595 +/- Donepezil effects on wake qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	3.101	0.0081	**	N=11-17	3 mg/kg donepezil vs Veh: 2Hz 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh: 2-3, 5-12 and 22-56Hz
						Frequency	79, 1264	13.62	<0.0001	****		
						Dose x Frequency	474, 5664	6.970	<0.0001	****		
7j	25-27mo	VU0453595 +/- Donepezil effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	5, 80	3.197	0.0111	*	N=7-17	30 mg/kg VU0453595 vs Veh Freq: 0.5-1.42 and 50-79 1mg/kg donepezil vs Veh Freq: 3Hz 30 mg/kg VU0453595 +1mg/kg donepezil vs Veh Freq: 2Hz 3 mg/kg donepezil vs Veh Freq: 3Hz
						Frequency	79, 1264	10.37	<0.0001	****		
						Dose x Frequency	395, 3920	2.461	<0.0001	****		
7k	25-27mo	VU0453595 +/- Donepezil effects on gamma power during wake	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	5.554	<0.0001	****	N=11-17	30 mg/kg VU0453595 vs Veh time: 0 and 1hr 30 mg/kg VU0453595 +1mg/kg donepezil vs Veh time: 0, 1and 2hr 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh time: 0, 1.2, 3 and 4hr
						Time	10, 160	42.19	<0.0001	****		
						Dose x Time	60, 696	7.639	<0.0001	****		
7l	25-27mo	VU0453595 +/- Donepezil effects on NREM delta (SWA) (-2 to 0 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 64	1.921	0.1177	ns	N=7-17	30 mg/kg VU0453595 vs Veh time: 0hr 3 mg/kg donepezil vs Veh time: 0hr
						Time	2, 32	15.47	<0.0001	****		
						Dose x Time	8, 71	3.499	0.0018	**		
	25-27mo	VU0453595 +/- Donepezil effects on NREM delta (SWA) (1 to 8 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	2.648	0.0159	*	N=7-17	30 mg/kg VU0453595 +1mg/kg donepezil vs Veh time: 1hr 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh time: 2hr
						Time	7, 112	10.55	<0.0001	****		
						Dose x Time	42, 419	2.894	<0.0001	****		
8a	3-4mo	VU0453595 effects on delta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	4.749	0.0500	*	N=13	None
						Time	10, 120	4.462	<0.0001	****		
						Dose x Time	10, 120	0.7818	0.6462	ns		
8b	19-20mo	VU0453595 effects on delta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.394	0.2590	ns	N=14	3 mg/kg vs Veh time: 1hr
						Time	10, 130	8.418	<0.0001	****		
						Dose x Time	30, 390	1.680	0.0155	ns		
8c	25-27mo	VU0453595+/-donepezil effects on delta power during wake	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	3.337	0.0050	**	N=11-17	30 mg/kg VU0453595 +1mg/kg donepezil vs Veh time: 0hr 3 mg/kg donepezil vs Veh time: 0, 1and 5hr 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh time: 0, 1and 2hr
						Time	10, 160	14.52	<0.0001	****		
						Dose x Time	60, 696	6.737	<0.0001	****		
8d	3-4mo	VU0453595 effects on theta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	1.587	0.2317	ns	N=13	N/A
						Time	10, 120	2.051	0.0338	*		
						Dose x Time	10, 120	1.439	0.1714	ns		
8e	19-20mo	VU0453595 effects on theta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.435	0.2473	ns	N=14	N/A
						Time	10, 130	4.020	<0.0001	****		
						Dose x Time	30, 390	1.094	0.3389	ns		
8f	25-27mo	VU0453595+/-donepezil effects on theta power during wake	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	10.91	<0.0001	****	N=11-17	3 mg/kg donepezil vs Veh time: 0, 1and 5hr 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh time: 0, 1.2 and 3hr
						Time	10, 160	22.65	<0.0001	****		
						Dose x Time	60, 696	21.63	<0.0001	****		
8g	3-4mo	VU0453595 effects on alpha power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	0.5881	0.6265	ns	N=14	10 mg/kg vs Veh Time: 3hr 30 mg/kg vs Veh Time: 0hr
						Time	10, 130	3.597	0.0003	***		
						Dose x Time	30, 390	2.515	<0.0001	****		
8h	19-20mo	VU0453595 effects on alpha power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	3.020	0.1078	ns	N=13	N/A
						Time	10, 120	10.69	<0.0001	****		
						Dose x Time	10, 120	0.7197	0.7197	ns		
8i	25-27mo	VU0453595+/-donepezil effects on alpha power during wake	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	19.25	<0.0001	****	N=11-17	30 mg/kg VU0453595 vs Veh time: 0hr 30 mg/kg VU0453595 +1mg/kg donepezil vs Veh time: 0 and 1hr 3 mg/kg donepezil vs Veh time: 0, 1and 5hr 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh time: 0, 1.2, 3.4 and 5hr
						Time	10, 160	51.81	<0.0001	****		
						Dose x Time	60, 696	14.56	<0.0001	****		
8j	3-4mo	VU0453595 effects on beta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.234	0.0325	*	N=14	10 mg/kg vs Veh Time: 0hr 30 mg/kg vs Veh Time: 0 and 1hr
						Time	10, 130	4.442	<0.0001	****		
						Dose x Time	30, 390	5.908	<0.0001	****		
8k	19-20mo	VU0453595 effects on beta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	1, 12	12.76	0.0038	**	N=13	30 mg/kg vs Veh Time: 0, 1.2 and 4hr
						Time	10, 120	10.85	<0.0001	****		
						Dose x Time	10, 120	4.611	<0.0001	****		
8l	25-27mo	VU0453595+/-donepezil effects on beta power during wake	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	2.936	0.0113	*	N=11-17	30 mg/kg vs Veh Time: 0 and 1hr 30 mg/kg VU0453595 +1mg/kg donepezil vs Veh time: 0hr 30 mg/kg VU0453595 +3 mg/kg donepezil vs Veh time: 1and 2hr
						Time	10, 160	6.969	<0.0001	****		
						Dose x Time	60, 696	3.176	<0.0001	****		

9a	3-4mo	VU0453595 effects on theta power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 12	0.03170	<0.0001	****	N=12-13	30 mg/kg vs Veh Time: 0hr
						Time	10, 120	7.956	0.8617	ns		
						Dose x Time	10, 96	3.874	0.0002	***		
9b	19-20mo	VU0453595 effects on theta power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.5431	0.6557	ns	N=12-14	N/A
						Time	10, 130	4.601	<0.0001	****		
						Dose x Time	30, 320	1.489	0.0517	ns		
9c	25-27mo	VU0453595 +/- Donepezil effects on NREM theta (-2 to 0 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 64	1.898	0.1216	ns	N=11-17	N/A
						Time	2, 32	29.88	<0.0001	****		
						Dose x Time	8, 71	1.991	0.0599	ns		
	25-27mo	VU0453595 +/- Donepezil effects on NREM theta (1 to 8 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	3.202	0.0066	**	N=11-17	1mg/kg donepezil vs Veh time: 6hr 30 mg/kg VU0453595 + 1mg/kg donepezil vs Veh time: 1hr 3 mg/kg donepezil vs Veh time: 1, 3, 4, 5 and 6hr 30 mg/kg VU0453595 + 3 mg/kg donepezil vs Veh time: 2 and 3hr
						Time	7, 112	3.546	0.0018	**		
						Dose x Time	42, 419	2.364	<0.0001	****		
9d	3-4mo	VU0453595 effects on alpha power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 12	0.4890	0.4977	ns	N=12-13	N/A
						Time	10, 120	3.995	<0.0001	****		
						Dose x Time	10, 96	0.7604	0.6661	ns		
9e	19-20mo	VU0453595 effects on alpha power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.250	0.3048	ns	N=12-14	N/A
						Time	10, 130	9.793	<0.0001	****		
						Dose x Time	30, 320	0.8457	0.7020	ns		
9f	25-27mo	VU0453595 +/- Donepezil effects on NREM alpha (-2 to 0 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 64	1.300	0.2797	ns	N=11-17	None
						Time	2, 32	60.61	<0.0001	****		
						Dose x Time	8, 71	2.631	0.0139	*		
	25-27mo	VU0453595 +/- Donepezil effects on NREM alpha (1 to 8 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	1.128	0.3518	ns	N=11-17	30 mg/kg VU0453595 + 1mg/kg donepezil vs Veh time: 1hr 3 mg/kg donepezil vs Veh time: 1, 2hr 30 mg/kg VU0453595 + 3 mg/kg donepezil vs Veh time: 3hr
						Time	7, 112	5.121	<0.0001	****		
						Dose x Time	42, 419	3.551	<0.0001	****		
9g	3-4mo	VU0453595 effects on beta power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 12	0.06209	0.8074	ns	N=12-13	30 mg/kg vs Veh Time: 0hr
						Time	10, 120	7.900	<0.0001	****		
						Dose x Time	10, 96	2.932	0.0053	**		
9h	19-20mo	VU0453595 effects on beta power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	2.128	0.1123	ns	N=12-14	N/A
						Time	10, 130	12.37	<0.0001	****		
						Dose x Time	30, 320	1.459	0.0611	ns		
9i	25-27mo	VU0453595 +/- Donepezil effects on NREM beta (-2 to 0 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 64	1.372	0.2534	ns	N=11-17	1mg/kg donepezil vs Veh time: -2hr 3 mg/kg donepezil vs Veh time: 0hr 30 mg/kg VU0453595 vs Veh time: 0hr
						Time	2, 32	9.396	0.0006	***		
						Dose x Time	8, 71	2.534	0.0174	*		
	25-27mo	VU0453595 +/- Donepezil effects on NREM beta (1 to 8 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	3.992	0.0013	**	N=11-17	10 mg/kg VU0453595 vs Veh time: 7hr 30 mg/kg VU0453595 + 1mg/kg donepezil vs Veh time: 1hr 3 mg/kg donepezil vs Veh time: 1hr 30 mg/kg VU0453595 + 3 mg/kg donepezil vs Veh time: 2hr
						Time	7, 112	6.444	<0.0001	****		
						Dose x Time	42, 419	2.777	<0.0001	****		
9j	3-4mo	VU0453595 effects on gamma power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 12	4.935	0.0463	*	N=12-13	30 mg/kg vs Veh Time: 0hr
						Time	10, 120	14.20	<0.0001	****		
						Dose x Time	10, 96	14.41	<0.0001	****		
9k	19-20mo	VU0453595 effects on gamma power during NREM	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.8487	0.4757	ns	N=12-14	N/A
						Time	10, 130	2.153	0.0247	*		
						Dose x Time	30, 320	1.492	0.0508	ns		
9l	25-27mo	VU0453595 +/- Donepezil effects on NREM gamma (-2 to 0 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 64	4.704	0.0022	**	N=11-17	30 mg/kg VU0453595 vs Veh time: 0hr 3 mg/kg donepezil vs Veh time: 0hr
						Time	2, 32	1.641	0.2096	ns		
						Dose x Time	8, 71	6.855	<0.0001	****		
	25-27mo	VU0453595 +/- Donepezil effects on NREM gamma (1 to 8 Hr)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	6, 96	1.529	0.1769	ns	N=11-17	10 mg/kg VU0453595 vs Veh time: 8hr 30 mg/kg VU0453595 vs Veh time: 1hr 30 mg/kg VU0453595 + 3 mg/kg donepezil vs Veh time: 2hr
						Time	7, 112	8.283	<0.0001	****		
						Dose x Time	42, 419	3.597	<0.0001	****		

10a	3-4mo	VU0453595 effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	24.94	0.0002	***	N=14	30 mg/kg vs Veh Freq: 4, 8-9, 44 and 46-79Hz
						Frequency	79, 1027	6.836	<0.0001	****		
						Dose x Frequency	79, 1027	11.19	<0.0001	****		
10b	3-4mo	VU0453595 effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	0.9281	0.3529	ns	N=14	30 mg/kg vs Veh Freq: 0.5 and 3Hz
						Frequency	79, 1027	6.002	<0.0001	****		
						Dose x Frequency	79, 1027	1.910	<0.0001	****		
10c	3-4mo	VU0453595 effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 13	0.1610	0.6947	ns	N=11-14	None
						Frequency	79, 1027	1.370	0.0207	*		
						Dose x Frequency	79, 787	1.565	0.0019	**		
10d	3-4mo	VU0453595 effects on gamma power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	9.790	0.0080	**	N=14	30 mg/kg vs Veh Time: 0, 1, 2 and 3hr
						Time	10, 130	20.12	<0.0001	****		
						Dose x Time	10, 130	6.111	<0.0001	****		
10e	3-4mo	VU0453595 effects on NREM delta (SWA)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1,13	0.1196	0.7350	ns	N=14	30 mg/kg vs Veh Time: 0hr
						Time	10, 130	40.76	<0.0001	****		
						Dose x Time	10, 129	3.277	0.0008	***		
10f	19-20mo	VU0453595 effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	5.314	0.0036	**	N=14	3 mg/kg vs Veh Freq: 1-2Hz 10 mg/kg vs Veh Freq: 16Hz 30 mg/kg vs Veh Freq: 0.5-1, 7-9, 11and 31-79hz
						Frequency	79, 1027	5.296	<0.0001	****		
						Dose x Frequency	237, 3081	3.314	<0.0001	****		
10g	19-20mo	VU0453595 effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.668	0.1896	ns	N=14	30 mg/kg vs Veh Freq: 0.5 and 39-79hz
						Frequency	79, 1027	2.825	<0.0001	****		
						Dose x Frequency	237, 3081	1.994	<0.0001	****		
10h	19-20mo	VU0453595 effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.639	0.1961	ns	N=13-14	10 mg/kg vs Veh Freq: 60, 62, 67-70, 72, 74-75 and 78-79Hz 30 mg/kg vs Veh Freq: 0.5-1, 3 and 37hz
						Frequency	79, 1027	2.954	<0.0001	****		
						Dose x Frequency	237, 3001	1.574	<0.0001	****		
10i	19-20mo	VU0453595 effects on gamma power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	1.972	0.1341	ns	N=14	3 mg/kg vs Veh Time: 7hr 10 mg/kg vs Veh Time: 2 and 7hr 30 mg/kg vs Veh Time: 1hr
						Time	10, 130	19.83	<0.0001	****		
						Dose x Time	30, 390	3.323	<0.0001	****		
10j	19-20mo	VU0453595 effects on NREM delta (SWA)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.3866	0.7632	ns	N=14	N/A
						Time	10, 130	26.95	<0.0001	****		
						Dose x Time	30, 386	1.061	0.3821	ns		
11a	3-4mo	VU0453595 effects on delta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	0.5921	0.4729	ns	N=14	30 mg/kg vs Veh Time: 1hr
						Time	10, 130	3.240	0.0006	***		
						Dose x Time	10, 130	3.000	0.0034	**		
11b	19-20mo	VU0453595 effects on delta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	2.538	0.0706	ns	N=14	10 mg/kg vs Veh Time: 2hr 30 mg/kg vs Veh Time: 1hr
						Time	10, 130	12.08	<0.0001	****		
						Dose x Time	30, 390	2.879	<0.0001	****		
11c	3-4mo	VU0453595 effects on theta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	1.609	0.2268	ns	N=14	30 mg/kg vs Veh Time: 0, 3 and 7hr
						Time	10, 130	5.359	<0.0001	****		
						Dose x Time	10, 130	3.689	0.0002	***		
11d	19-20mo	VU0453595 effects on theta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	3.627	0.0211	*	N=14	3 mg/kg vs Veh Time: 6hr 30 mg/kg vs Veh Time: 1hr
						Time	10, 130	3.330	0.0007	***		
						Dose x Time	30, 390	2.258	0.0002	***		
11e	3-4mo	VU0453595 effects on alpha power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	5.352	0.0377	*	N=14	30 mg/kg vs Veh Time: 1, 2 and 5hr
						Time	10, 130	3.945	0.0001	***		
						Dose x Time	10, 130	3.747	0.0002	***		
11f	19-20mo	VU0453595 effects on alpha power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	0.6065	0.6148	ns	N=14	30 mg/kg vs Veh Time: 0, 1and 2hr
						Time	10, 130	6.582	<0.0001	****		
						Dose x Time	30, 390	2.821	<0.0001	****		
11g	3-4mo	VU0453595 effects on beta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	1, 13	2.970	0.1085	ns	N=14	N/A
						Time	10, 130	4.299	<0.0001	****		
						Dose x Time	10, 130	1.046	0.4094	ns		
11h	19-20mo	VU0453595 effects on beta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	2.215	0.1017	ns	N=14	10 mg/kg vs Veh Time: 0, 2 and 6hr 30 mg/kg vs Veh Time: 0 and 6hr
						Time	10, 130	9.811	<0.0001	****		
						Dose x Time	30, 390	2.576	<0.0001	****		

12a	3-4mo	VU0453595 effects on theta power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 13	0.006016	0.9394	ns	N=14	30 mg/kg vs Veh Time: 0hr
						Time	10, 130	30.52	<0.0001	****		
						Dose x Time	10, 129	2.737	0.0044	**		
12b	19-20mo	VU0453595 effects on theta power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.1766	0.9116	ns	N=14	N/A
						Time	10, 130	22.32	<0.0001	****		
						Dose x Time	30, 386	0.5259	0.9826	ns		
12c	3-4mo	VU0453595 effects on alpha power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 13	0.8107	0.3843	ns	N=14	N/A
						Time	10, 130	30.80	<0.0001	****		
						Dose x Time	10, 129	1.108	0.3609	ns		
12d	19-20mo	VU0453595 effects on alpha power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.642	0.1953	ns	N=14	30 mg/kg vs Veh Time: 0 and 1hr
						Time	10, 130	50.29	<0.0001	****		
						Dose x Time	30, 386	1.961	0.0023	**		
12e	3-4mo	VU0453595 effects on beta power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 13	1.051	0.3420	ns	N=14	30 mg/kg vs Veh Time: 0hr
						Time	10, 130	24.34	<0.0001	****		
						Dose x Time	10, 129	2.454	0.0102	*		
12f	19-20mo	VU0453595 effects on beta power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	0.4141	0.7438	ns	N=14	N/A
						Time	10, 130	26.46	<0.0001	****		
						Dose x Time	30, 386	1.295	0.1409	ns		
12g	3-4mo	VU0453595 effects on gamma power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 13	6.165	0.0275	*	N=14	30 mg/kg vs Veh Time: 0 and 1hr
						Time	10, 130	8.688	<0.0001	****		
						Dose x Time	10, 129	8.857	<0.0001	****		
12h	19-20mo	VU0453595 effects on gamma power during NREM	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	1.029	0.3903	ns	N=14	30 mg/kg vs Veh Time: 0hr
						Time	10, 130	5.019	<0.0001	****		
						Dose x Time	30, 386	5.425	<0.0001	****		
13a	Comparison	PFC Cortical Thickness	Direct Comparison	N/A	One- Way ANOVA	Age	2, 22	0.1229	0.8850	ns	N=7-9	N/A
13b	Comparison	PFC fiber density	Direct Comparison	N/A	One- Way ANOVA	Age	2, 22	0.1556	0.8569	ns	N=7-9	N/A
13c	Comparison	PFC fiber density (superficial layers)	Direct Comparison	N/A	One- Way ANOVA	Age	2, 22	0.1876	0.8303	ns	N=7-9	N/A
13d	Comparison	PFC fiber density (deep layers)	Direct Comparison	N/A	One- Way ANOVA	Age	2, 22	0.2423	0.7869	ns	N=7-9	N/A

Table 4.2: Detailed statistical analysis.

CHAPTER 5

Activation of the M_4 Muscarinic Acetylcholine Receptor with the M_4 Positive Allosteric Modulator VU0467154 Modulates Sleep/Wake Architecture in Young and Non-pathologically Aged Mice.

5.1. Introduction

Accumulating evidence suggests that cholinergic signaling is vitally important in promoting wakefulness, arousal, and rapid eye movement (REM) sleep (Han et al., 2014; B. E. Jones, 2020; M. G. Lee et al., 2005; Xu et al., 2015). Specifically, the muscarinic acetylcholine receptors (mAChR) are believed to play a fundamental role in modulating sleep/wake architecture (Brown et al., 2012). There are five muscarinic acetylcholine receptor (mAChR) subtypes (M_1-M_5) (Bonner et al., 1987, 1988), of which the M_1, M_2 and M_3 mAChRs have been suggested to regulate rapid eye movement (REM) sleep (Coleman et al., 2004; Gould et al., 2020; Niwa et al., 2018) and the M_1 mAChR has been implicated in wakefulness and arousal (Gould et al., 2020).

The effects of the M_4 mAChR on sleep/wake architecture have been poorly studied to date. Historical studies have suggested that M_4 mAChR knock-out either has no effect on sleep/wake cycle or REM sleep in basal conditions or following sleep deprivation (Goutagny et al., 2005; Turner et al., 2010), or produces small reductions in total sleep time over 24 hours (Niwa et al., 2018). With knock-out studies there is the potential that compensatory mechanisms could mask a potential role for the M_4 mAChR in sleep/wake architecture. The development of highly selective M_4 mAChR positive allosteric modulators (PAMs), which target less highly conserved allosteric sites on the receptor, to increase subtype selectivity, typified by the highly selective M_4 mAChR PAM tool compound VU0467154 (Bubser et al., 2014) allows for the investigation of the

effects of M_4 mAChR activation on sleep/wake architecture and arousal. Furthermore, the recent development of highly selective orthosteric M_4 mAChRs enables us to test whether the observed effects are M_4 mAChR dependent.

Given the role of the M_4 mAChR as a cholinergic autoreceptor (Tzavara et al., 2003)., we hypothesize that increased stimulation of the M_4 mAChR would reduce states dependent on cholinergic signaling, specifically wakefulness, arousal and REM sleep. Previous work from our lab in rats has suggested that M_4 activation following dosing with the M_4 PAM VU0467154 produces increased latency to REM sleep, decreased REM sleep duration and increased total sleep over 24 hours following dosing in preclinical species (Gould et al., 2016). However, in these studies these effects were not confirmed to be M_4 mediated through knock-out or pharmacological reversal. Additional work by our group has shown that M_4 mAChR stimulation improves learning and memory with dosing both prior to and post training (Bubser et al., 2014; Gould et al., 2018), suggesting a potential M_4 mAChR mediated effect on memory consolidation. It has been demonstrated that low levels of acetylcholine during NREM sleep is important for memory consolidation (Gais and Born, 2004; Inayat et al., 2020), which would be consistent with M_4 mAChRs role as an autoreceptor.

Currently, both PAM and orthosteric mechanisms targeting the M_4 mAChR are progressing through clinical development for the treatment of schizophrenia and/or the behavioral disturbances associated with Alzheimer's Disease (AD) (Brannan et al., 2021)(ClinicalTrials.gov: NCT03697252, NCT04659161, NCT05511363, NCT05227690). The efficacy of PAMs is dependent on the endogenous ligand, which maintains the spatial and temporal specificity of endogenous signaling (Conn, Jones, et al., 2009). In the case of acetylcholine, concentrations of the endogenous ligand is reduced with advancing age (Mitsushima et al., 1996; C. F. Wu et al., 1988). We have previously demonstrated the antipsychotic and cognitive-enhancing effects of M_4 mAChR PAMs in young mice (Bubser et al., 2014; Gould et al., 2018), and state-dependent effects of the M_4 mAChR PAM VU0467154 on sleep/wake architecture in young rats (Gould et

al., 2016). This will be the first assessment of M_4 mAChR PAMs in aging where central cholinergic integrity decreases (Bartus et al., 1982; Dumas and Newhouse, 2011), which may influence M_4 mAChR PAM efficacy. Further, prefrontal cortical cholinergic signaling is typically high during active periods (lights off for rodents), and low during inactive periods with greater sleep (lights on for rodents) (Mitsushima et al., 1996), as such assessments will be performed across the diurnal rhythm.

The current study systematically assesses the effects of the M_4 mAChR PAM VU0467154 on sleep/wake architecture and qEEG in young mice. To confirm the observed effects are M_4 mAChR mediated we used the selective M_4 mAChR antagonist VU6028418 to attenuate observed effects. Further, we investigated whether these M_4 mediated effects on sleep/wake architecture are also observed in non-pathologically mice. In addition, this study evaluates the side effect profile of VU0467154 and VU0467154 exposure relative to efficacy in non-pathologically aged mice. These data provide the first definitive evidence for the role for M_4 mAChR stimulation in the control of NREM sleep quality and/or quantity and REM sleep quantity in young and non-pathologically aged mice. The effects observed on sleep/wake architecture provide support for M_4 mAChR PAMs in the treatment of schizophrenia and reveal some important considerations for the utility of M_4 mAChR PAMs in AD.

5.2. Methods

Subjects

Young adult (4-5-month-old, n=29, n=15 for VU0467154 alone EEG studies and n=14 for VU0467154 in combination with VU6028418 EEG studies) and non-pathologically aged (20-21-month, n=34; n=15 for EEG, n=11 for Modified Irwin, n=8 for pharmacokinetic (pK) studies) wildtype male C56BL/6J mice (The Jackson Laboratory) served as subjects. Due to age being an experimental variable and the mechanisms of menopause and estropause being different between mice and clinical populations (Carolino et al., 2019), future studies with muscarinic

ligands will assess the role of aging and estrogen loss in female rodents. All animals were socially housed prior to study initiation. Mice lived in temperature and humidity-controlled rooms maintained under a 12/12hr light-dark cycle with access to food and water *ad libatum*. Following implantation of EEG devices all animals were individually housed. All studies 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*.

Compounds

The M_4 mAChR PAM VU0467154 and the M_4 mAChR antagonist VU6028418 were synthesized in house (Bubser et al., 2014; Spock et al., 2021). For VU0467154 alone studies VU046154 was formulated in a microsuspension in 10% tween 80 in sterile water, for combination studies VU0467154 and VU6028418 were formulated in 5% tween 80 in sterile water. Following formulation, the final pH was adjusted to 6-7. VU0467154 and VU06028418 were administered intraperitoneally (i.p.) at 10 mL/kg for EEG (both compounds), Modified Irwin and pharmacokinetic (pK) assessments (only VU0467154). The VU0467154 dose range used for EEG (1.0 – 30 mg/kg) has previously been shown to produce cognitive enhancement (Bubser et al., 2014; Gould et al., 2018) in young mice and rats, and alter sleep/wake architecture in young rats (Gould et al., 2016). For combination studies the highest dose of VU0467154 was selected with 10 mg/kg of VU6028418, a dose predicted produce total brain concentrations significantly greater than the compounds IC_{50}.

Electroencephalography

Surgery. All mice used for sleep assessment were surgically implanted under isoflurane anesthesia with a telemetric transmitter (HD-X02, Data Science International [DSI], Minneapolis, MN) as previously described (Fisher et al., 2020; Gould et al., 2020). Using aseptic technique a 2-3cm midline incision was made over the scull. For recording EEG a frontoparietal lead was placed at +1.5 mm AP, -2 mm ML and -3 mm AP, 2 mm ML and secured with screws and covered

with dental cement (Patterson Dental, Saint Paul, MN). A second biopotential lead was placed in the nuchal muscle for electromyogram (EMG) recording. Animals were allowed to recover for a minimum of 10-days prior to recording.

EEG recording. EEG and EMG were recorded in the home cage of each animal, with recordings being performed for 24-hours starting at either lights on (for inactive phase dosing) or lights off (for active phase dosing). EEG and EMG waveforms were captured using Ponemah software (v3.0, DSI). Data was continuously sampled at 500Hz and transmitted by a wireless receiver (RPC-1, DSI) below each mouses home cage. Dosing with VU0467154 (1.0 – 30 mg/kg, or tween 80 vehicle i.p.) was performed 2-3hours into the relevant phase of the light cycle to allow assessment of the compound effects when the animals are active and inactive. For combination studiesthe M_4 mAChR antagonist VU6028418 was dosed 2-2.5 hours into the phase of interest with VU0467154 dosed 30 minutes later, to allow sufficient uptake of VU6028418 before administering VU0467154. All experiments are displayed in zeitgeber time, where ZT0 indicates transition from the active (dark) into the inactive (light) phase.

Sleep staging and analysis. Trained observers blinded to age and dose manually scored 5-second epochs using Neuroscore 3.3.1 software (DSI) as wake, NREM or REM based on accepted characteristic oscillatory patterns as previously published by our group (Fisher et al., 2020; Gould et al., 2016, 2020; Nedelcovych et al., 2015). The amount of time in each state (wake, NREM, REM) in 2-hour bins across the 24-hour recording served as the primary dependent measure to determine the effects of age and pharmacologic challenge. Sleep fragmentation was assessed by measuring average NREM bout duration and number of NREM bouts for 8 hours following dosing. REM sleep latency was assessed by the duration of time in seconds from dosing until the first epoch of REM sleep

qEEG spectral power analysis. Following sleep staging, quantitative EEG (qEEG) relative power spectra were computed in 1Hz bins from 0.5 to 80Hz using a Fast Fourier Transformation with a Hamming window overlap ratio of 0.5. Relative spectral power within each 1Hz interval was

then grouped by stage (wake, NREM or REM) and averaged across a 1-hour predose baseline 1-2 hours after the light change and a 1-hr post dose period from 1 to 2-hours post dose. The 1-hour post-dose period is then represented within wake state as a percent change relative to the predose period. For the assessments of spectral power change over time within a power band, the relative spectral power within a power band was averaged in 1-hour bins for 8 hours following dosing with VU0467154 for studies with VU0467154 alone and in combination with VU6028418 and displayed relative to the 1-hour predose baseline. For age-dependent changes the 1-hour predosing baseline period on vehicle dosing days is used for spectral power comparisons across the full spectogram. For gamma and delta power comparisons across a phase the aged mouse data is normalized to the corresponding time in the young mouse data.

Assessing unconditioned behavioral effects

The effects of VU0467154 on autonomic and somatomotor function in aged C57BL/6J mice were assessed (n=5-6 per group) using the modified Irwin testing battery (Irwin, 1968). Previously, we demonstrated 10 and 30 mg/kg have no effect in young mice (Bubser et al., 2014). In the present study, assessments were performed 30, 60,120 and 240 min after i.p. administration of 30 mg/kg VU0467154 or 10% tween 80 in sterile water. Observers blinded to condition scored mice as 0-normal, 1-mild effect and 2-marked effect on a battery of behavioral endpoints (see Table 5.5 for full list of behaviors assessed).

Pharmacokinetics study

VU0467154 was formulated as previously described and dosed i.p. in aged C57BL/6J mice at 3 mg/kg to allow comparison with our previously published young animal data (Bubser et al., 2014). Brain and plasma were collected at 1-hour and 4-hours post dosing non-serially (n=3-4 per time). Brain and plasma concentrations were quantified by electrospray ionization using an AB Sciex Q-TRAP 5500 (Foster City, CA) that was coupled to a Shimadzu LC-20AD pump (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a C18 column (3 x 50 mm, 3 mm; Fortis Technologies Ltd,

Cheshire, UK) that was thermostated at 40°C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted); mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). A 10% B gradient was held for 0.2 minute and was linearly increased to 90% B over 0.8 minute, with an isocratic hold for 0.5 minute, before transitioning to 10% B over 0.05 minute. The column was re-equilibrated (1 minute) before the next sample injection. The total run time was 2.55 minutes, and the HPLC flow rate was 0.5 ml/min. The source temperature was set at 500°C, and mass spectral analyses were performed using a Turbo-Ion spray source in positive ionization mode (5.0-kV spray voltage) and using multiple-reaction monitoring of transitions specific for the analyte (m/z 445.0 to 179.0 at 47 eV). All data were analyzed using AB Sciex MultiQuant 2.2 software. The lower limits of quantitation for VU0467154 was determined at 0.5 ng/ml in plasma and in brain homogenates. Brain-to-plasma ratios were calculated using a single time point and free brain concentrations were compared to previously published data in young animals (Bubser et al., 2014).

Data and Statistical Analysis

GraphPad Prism 9.4.1 was used for all statistical analyses and graphing. Sleep/wake architecture and qEEG are represented as means ± S.E.M. in either 1-hr, 2-hr or 12-hr bins, or in 1hz intervals. Two-way repeated measures analysis of variance (repeating both factors for dosing studies, repeated by one factor for young to aged comparisons) was used in all qEEG and sleep/wake assessments, except for the NREM qEEG analyses in the active phase and REM qEEG analysis when some mice did not NREM or REM sleep respectively during the analyzed period, so a repeated measures mixed effects model (REML) was applied. For comparisons of NREM and wake sleep bout number and NREM and wake sleep bout duration repeated measures one-way analysis of variance was used. For all analyses post hoc tests were performed by a Dunnett's test for comparison to vehicle group, or Sidaks test for comparison between several dose groups when appropriate (see Table 5.6 for full statistical analysis). For all experiments statistical significance was defined as $p<0.05$. For all EEG studies n=14 mice were instrumented

as 12-14 animals has been sufficient to display robust statistical effects in previous work by our group (Gould et al., 2020). For young to aged comparisons only mice who were examined in both phases were used resulting in n=13 young mice in each phase. For latency to REM sleep in the aged group one mouse displayed a greatly increased REM latency which was identified as an outlier following a Grubbs outlier test (Alpha = 0.0001) and was removed from all analyses.

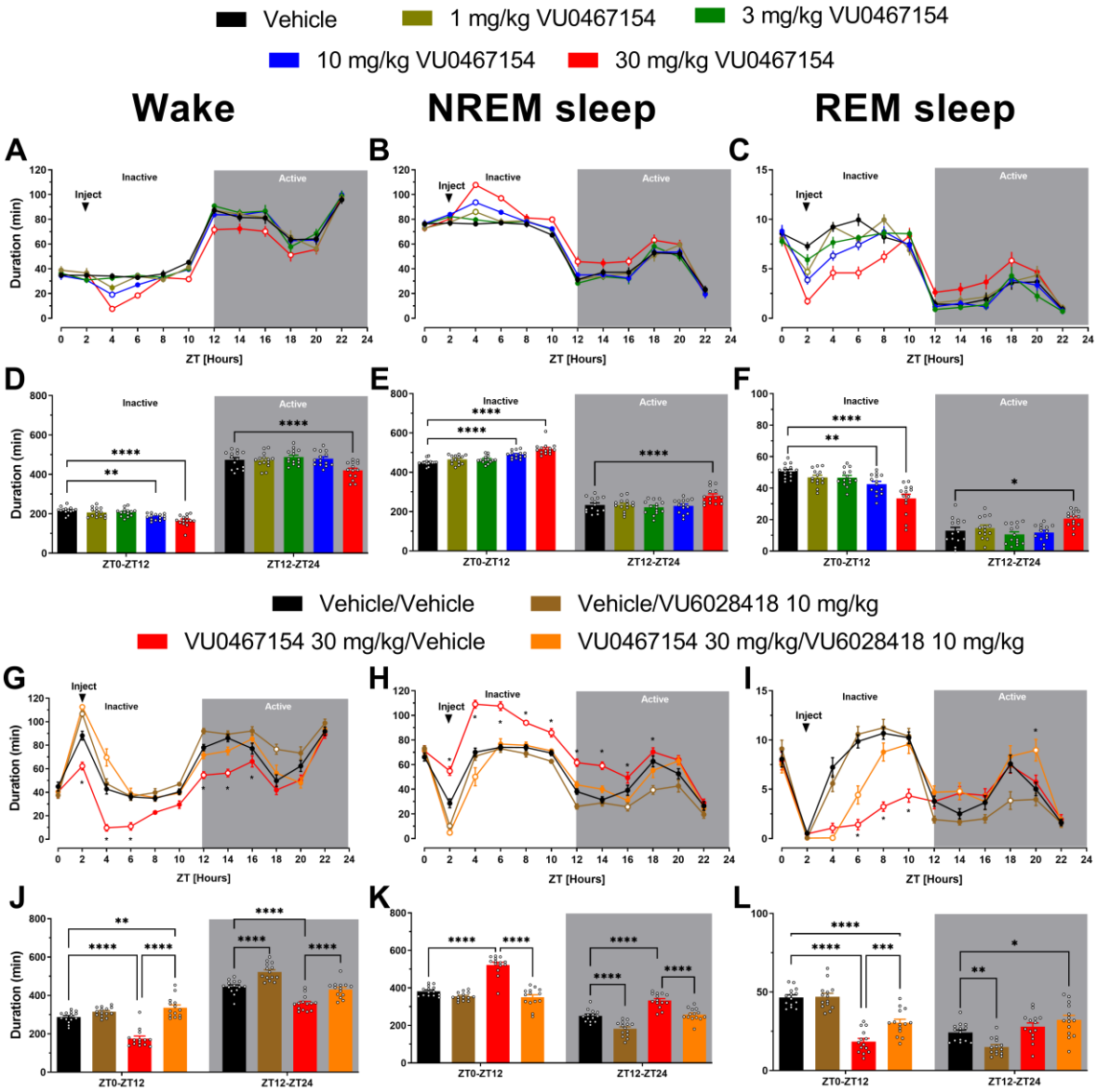

Figure 5.1. The M_4 mAChR PAM VU0467154 produced dose dependent increases in NREM sleep and reduced REM sleep during the inactive phase in young mice, which was attenuated by VU6028418. Shown is the duration of time in wake (A, D), NREM sleep, (B, E) and REM sleep (C, F) in young mice (4-5-month) following VU0467154 administration 2 hours into the inactive period (A-F) or VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change (G-L) (see arrowhead). In young mice, 10 and 30 mg/kg VU0467154 decreased duration of time in wake following dosing (A), with decreased wake over 12 hours observed in the inactive phase at 10 and 30 mg/kg with extends into the active phase at 30 mg/kg (D). 1, 10 and 30 mg/kg VU0467154 increased duration of time in NREM sleep following dosing (B), with decreased NREM sleep observed across the 12 hours of the inactive phase at 10 and 30 mg/kg, which extends into the 12 hours of the active phase at 30 mg/kg (E). 1, 10 and 30 mg/kg VU0467154 decreased duration of time in REM following dosing (C), with decreased REM sleep seen over the 12 hours of the inactive phase at 10 and 30 mg/kg, with a rebound increase in REM sleep seen in the 12 hours of the active phase at 30 mg/kg (F). Following dosing in the inactive phase with 30 mg/kg, VU0467154 again reduced wake (G, J), increased NREM sleep (H, K) and reduced REM sleep (I, L). VU6028418 dosed at 10 mg/kg produced transiently increased wakefulness (G, J) and reduced NREM sleep (H, K), with a reduction in REM sleep at ZT18 and ZT12-24 (I, L). 30mg/kg VU0467154 dosed in combination with 10 mg/kg VU6028418 blocked the previously observed reduced wake (G, J), and increased NREM (H, K) and partially blocked the reduced REM sleep (I, L) seen following 30 mg/kg VU0467154 dosing. Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle, and * indicate 30 mg/kg VU0467154 $p<0.05$ compared to 30 mg/kg VU0467154 with 10 mg/kg VU6028418 (2-way ANOVA matching by both factors followed by Dunnett's (A-C) or Sidak's (G-I) test); or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ compared to vehicle or 30 mg/kg VU0467154 compared to 30 mg/kg VU0467154 with 10 mg/kg VU6028418 (RM 1-way ANOVA followed by Dunnett's (D-F) or Sidak's (J-L) test), n=14/group; See table 5.6 for full statistical analysis.

5.3. Results

The M_4 mAChR PAM VU0467154 dosed in the inactive phase increased NREM sleep and reduced REM sleep in young mice, which was attenuated by the M_4 mAChR antagonist VU6028418.

In young mice, VU0467154 dose dependently reduced time in wake, 10 mg/kg produced a transient reduction in wake, and 30 mg/kg produced a larger more prolonged reduction in wake which extended into the active phase (Figure 5.1A), when assessed across the 12 hours of the inactive and active phases a reduction in wake is seen at 10 and 30 mg/kg which extends into the active phase at 30 mg/kg (Figure 5.1D). Consistent with this reduction in wake, VU0467154 dose dependently increased time in NREM sleep with 1 and 10 mg/kg producing transient increases in NREM sleep and 30 mg/kg producing a greater more prolonged increase in NREM sleep (Figure 5.1B), when assessed across 12 hours, 10 and 30 mg/kg produced increased NREM with the effects at 30 mg/kg seen across the inactive and active phase (Figure 5.1E). VU0467154 produced reductions in REM sleep following dosing. 1 mg/kg and 10 mg/kg both produced transient reductions in REM sleep, with 30 mg/kg producing a larger more prolonged reduction in

REM sleep that then rebounded during the active phase (Figure 5.1C), when assessed across 12 hours, 10 and 30 mg/kg produced decreased REM with 30 mg/kg resulting in a rebound increase in REM during the active phase (Figure 5.1F).

Similar to previously described effects, 30 mg/kg VU0467154 produced decreased wake and REM sleep, with decreased NREM sleep when dosed to young mice in the inactive phase. The M_4 mAChR antagonist VU6028418 blocked the decrease in wake when dosed in combination with VU0467154 and produced only transient wake promoting effects when dosed alone (Figure 5.1G). When assessed across in the 12 hours of the inactive period VU6028418 blocked the effects of VU0467154 but had no effect when dosed alone (Figure 5.1J). VU6028418 blocked the NREM promoting effects of VU0467154 whether assessing in 2-hour bins (Figure 5.1H) or 12-hour bins (Figure 5.1K). VU6028418 dosed with VU0467154 produced a partial blockade of the reduced REM sleep, as can be seen in 2-hour bins (Figure 5.1I) and 12-hour bins (Figure 5.1L).

The M_4 mAChR PAM VU0467154 decreases NREM sleep fragmentation and increased REM sleep latency in young mice when dosed in the inactive phase, with effects on NREM sleep fragmentation attenuated by the M_4 mAChR antagonist VU6028418.

In young mice VU0467154 displayed a main effect on NREM bout number and NREM bout duration. 10 and 30 mg/kg decreased NREM bout number and increased NREM bout duration (Figure 5.2A, B). This reduction in fragmentation results in dose dependent reductions in wake bout number during the inactive phase in young mice with significant reductions at 3, 10 and 30 mg/kg. Wake bout duration displays an overall effect of VU0467154 dose in the inactive phase, however no effect is seen at any individual dose following posthoc comparisons (Table 5.1).

VU0467154 and VU6028418 combination dosing produced a main effect of dose on NREM sleep bout number, with increased NREM sleep bout number observed following dosing with 10 mg/kg of the M_4 mAChR antagonist VU6028418 alone and in combination with 30 mg/kg VU0467154 during the inactive phase (Figure 5.2D). 30 mg/kg VU0467154 produced an increase

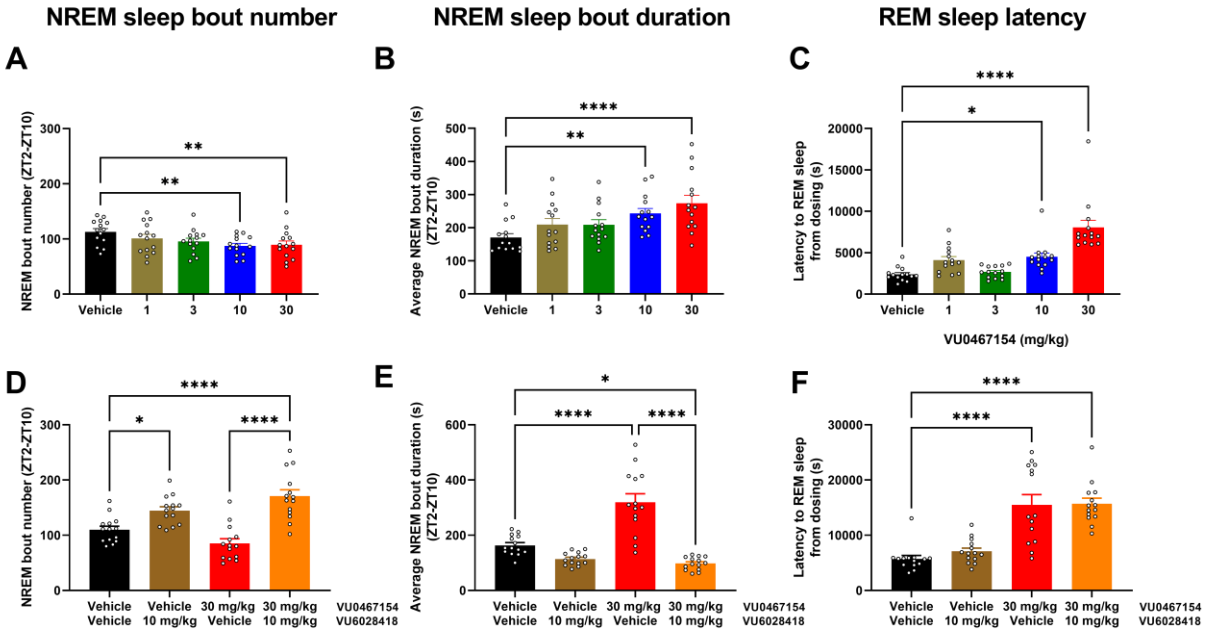

Figure 5.2. The M_4 PAM VU0467154 produced dose dependent reductions in NREM sleep fragmentation and increased REM sleep latency during the inactive phase in young mice, with the effects on fragmentation attenuated by VU6028418. Shown is the average NREM sleep bout number (A, D), the average NREM sleep bout duration (B, E) for 8 hours following dosing, and latency to REM sleep (C, F) in young mice during the inactive phase. 10 and 30 mg/kg VU04567154 decreased NREM sleep bout number and increased NREM sleep bout duration in young mice (A, B), effects which are attenuated by VU6028418 (C, D). VU0467154 10 and 30 mg/kg increased latency to REM sleep in young mice following dosing in the inactive phase (C), which was not attenuated by VU6028418 (F). Data are expressed as overall means ± S.E.M., n=14/group. * indicates p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 compared to vehicle, or 30 mg/kg VU0467154 compared to 30 mg/kg VU0467154 with 10 mg/kg VU6028418 (RM 1-way ANOVA followed by Dunnett's (A-C) or Sidak's (D-F) test). See table 5.6 for full statistical analysis.

in NREM sleep bout duration in the inactive phase, which was reversed following combination dosing with the M_4 mAChR antagonist 10 mg/kg VU6028418 (Figure 5.2E).

Following VU0467154 and VU06028418 combination dosing studies during the inactive phase, a 30 mg/kg VU0467154 produced no significant effect on wake bout number during the inactive phase following posthoc comparisons, although a trend towards a decrease, similar to previous data, was observed. The M_4 mAChR antagonist VU06028418 dosed at 10 mg/kg increased wake bout number with 30 mg/kg VU0467154 dosed with 10mg/kg VU6028418 producing a similar effect to VU6028418 alone. 30 mg/kg VU0467154 produced a reduction in wake bout duration with no effect observed following dosing with 10 mg/kg of the M_4 mAChR antagonist VU6028418 alone or in combination with 30 mg/kg VU0467154 (Table 5.1).

Table 5.1. Wake bout number and duration following dosing with VU0467154 alone or in combination with VU6028418. Shown is the average wake bout number and average wake bout duration in the inactive and active phases in young and non-pathologically aged mice for 8 hours following VU0456154 dosing 2 hours after light change. Data are expressed as overall means ± S.E.M., n=14/group. * indicates p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 compared to vehicle, ## indicates 10 mg/kg VU6028418/30 mg/kg VU0467154 p<0.01 and #### p<0.0001 compared to Vehicle/30 mg/kg VU0467154 (RM 1-way ANOVA followed by Dunnett's or Sidak's test).

Inactive (ZT2-ZT10)							
VU0467154 (mg/kg)	**Vehicle**	**1**	**3**	**10**	**30**	**2-Way ANOVA**	**P value**
Wake Bout Number (SEM)	114.4 (5.55)	100.9 (7.56)	95.86 (5.64)*	86.00 (4.78)***	87.43 (7.13)**	$F_{4,52}$ = 5.218	0.0013
Average Wake Bout Duration (s) (SEM)	73.54 (2.97)	75.94 (5.05)	85.27 (4.56)	79.56 (4.81)	61.49 (5.45)	$F_{4,52}$ = 5.088	0.0015
VU0467154 (mg/kg)	**Vehicle**	**Vehicle**	**30**	**30**			
VU6028418 (mg/kg)	**Vehicle**	**10**	**Vehicle**	**10**		**2-Way ANOVA**	**P value**
Wake Bout Number (SEM)	109.1 (5.754)	126.9 (9.859)**	93.21 (4.945)	129.6 (6.155)*/####		$F_{3,39}$ = 25.52	<0.0001
Average Wake Bout Duration (s) (SEM)	87.92 (5.039)	73.64 (3.938)	51.95 (4.235)***	70.58 (7.896)		$F_{3,39}$ = 8.032	0.0003
Active (ZT14-ZT22)							
VU0467154 (mg/kg)	**Vehicle**	**1**	**3**	**10**	**30**	**2-Way ANOVA**	**P value**
Wake Bout Number (SEM)	95.21 (6.43)	109.9 (6.78)	93.29 (7.40)	86.64 (6.96)	87.43 (5.39)	$F_{4,52}$ = 3.613	0.0113
Average Wake Bout Duration (s) (SEM)	198.3 (18.94)	145.9 (10.4)**	182.2 (17.5)	174.6 (16.8)	106.7 (7.83)****	$F_{4,52}$ = 11.56	<0.0001
VU0467154 (mg/kg)	**Vehicle**	**Vehicle**	**30**	**30**			
VU6028418 (mg/kg)	**Vehicle**	**10**	**Vehicle**	**10**		**2-Way ANOVA**	**P value**
Wake Bout Number (SEM)	109.1 (21.53)	126.9 (36.89)	93.21 (18.50)	129.6 (6.155)##		$F_{3,39}$ = 6.178	0.0015
Average Wake Bout Duration (s) (SEM)	168.4 (8.964)	179 (18.61)	117.3 (16.98)	147.4 (8.343)		$F_{3,39}$ = 3.542	0.0232

In young mice dosed during the inactive phase a dose dependent reduction in REM sleep latency is observed, with significantly increased REM sleep latency seen at 10 and 30 mg/kg (Figure 5.2C). When dosed in the inactive phase in the VU0467154 and VU6028418 co-dosing studies, 30 mg/kg VU0467154 produced a significant reduction in REM sleep latency. This is not reversed following combination dosing with 10 mg/kg VU6028418. 10 mg/kg VU6028418 dosed alone produced no effect on REM latency (Figure 5.2F).

The M_4 mAChR PAM VU0467154 dosed in the active phase increased NREM sleep in young mice, which was attenuated by the M_4 mAChR antagonist VU6028418.

In young mice, VU0467154 dose-dependently reduced time in wake. 1 and 3 mg/kg produced similar reductions in wake, with 10 and 30 mg/kg producing greater, more prolonged reductions in wake (Figure 5.3A). VU0467154 produced an increase in NREM sleep following dosing. When assessing time spent in wake across 12 hours, all doses tested produce decreased time in wake, while 30 mg/kg also produced a rebound increased time in wake during the active phase (Figure 5.3D). 1 and 3 mg/kg produced short increases in time in NREM with 10 and 30 mg/kg increasing the effect size and duration (Figure 5.3B), when assessing across 12 hours all

doses increased NREM sleep during the active phase (Figure 5.3E). In contrast to the inactive phase dosing, which reduced REM sleep, active phase dosing produced modest increases in REM sleep at 1 and 30 mg/kg following dosing, however 10-18-hours following dosing reductions in REM sleep are seen at 3, 10 and 30 mg/kg (Figure 5.3C), when assessing effects on REM sleep across 12 hours, 30 mg/kg produced increased REM sleep during the active phase, while 10 and 30 mg/kg produced reduced REM sleep in the inactive phase 12 hours after dosing (Figure

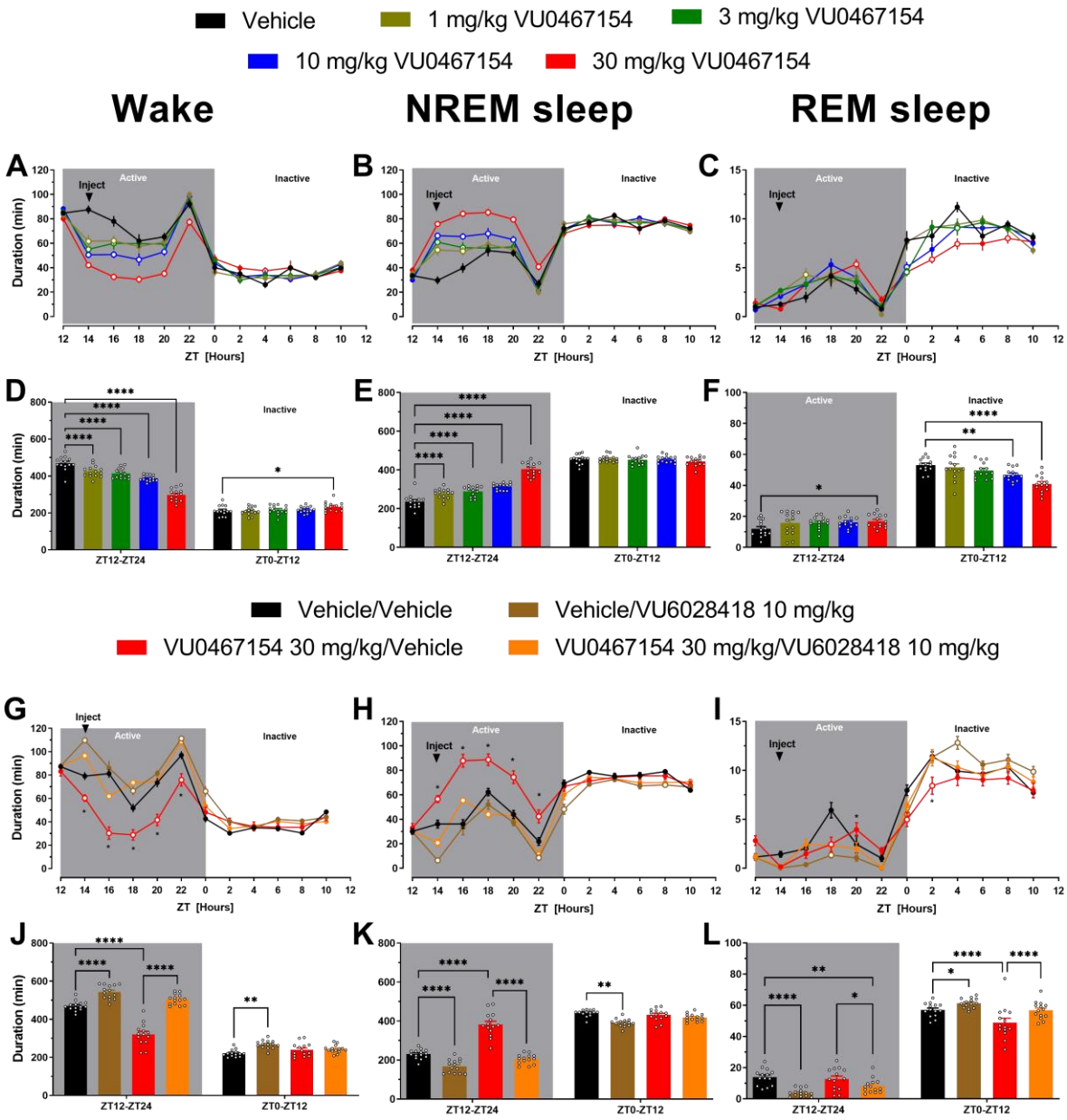

Figure 5.3. The M_4 mAChR PAM VU0467154 produced dose dependent increases in NREM sleep during the active phase in young mice, which was attenuated by VU6028418. Shown is the duration of time in wake (A, D), NREM sleep, (B, E) and REM sleep (C, F) in young mice (4-5-month) following VU0467154 administration 2 hours into the active period (A-F) or VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change (G-L) (see arrowhead). In young mice, 1, 3, 10 and 30 mg/kg VU0467154 decreased duration of time in wake following dosing (A), with reduced wake seen across the 12 hours of the active period at 1, 3, 10 and 30 mg/kg and a rebound increase in wake seen during the inactive period at 30 mg/kg (D). 1, 3, 10 and 30 mg/kg VU0467154 increased duration of time in NREM sleep following dosing (B), with increased NREM sleep seen across the 12 hours of the active period at 1, 3, 10 and 30 mg/kg (E). 1 mg/kg and 30 mg/kg VU0467154 increased duration of time in REM sleep following dosing, with 3, 10 and 30 mg/kg reducing REM sleep into the inactive phase (C); over the 12 hours of the active phase 30 mg/kg increased REM sleep, with 10 and 30 mg/kg reducing REM sleep across the 12 hours of the inactive phase (F). Following dosing in the active phase with 30 mg/kg, VU0467154 produced reduced wake (G, J) and increased NREM sleep (H, K), additionally reduced REM sleep in the 12-24 hours following dosing (I, L) was observed. VU6028418 dosed at 10 mg/kg produced increased wakefulness (G, J) and reduced NREM sleep (H, K), with a reduction in REM sleep at from ZT12-24, and increased REM sleep from ZT0-12 (I, L). 30 mg/kg VU0467154 dosed in combination with 10 mg/kg VU6028418 blocked the previously observed reduced wake (A, D), and increased NREM (B, E) and the reduced REM sleep from ZT0-12 (C, F) seen following 30 mg/kg VU0467154 dosing. Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle, and * indicate 30 mg/kg VU0467154 $p<0.05$ compared to 30 mg/kg VU0467154 with 10 mg/kg VU6028418 (2-way ANOVA matching by both factors followed by Dunnett's (A-C) or Sidak's (G-I) test); or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ compared to vehicle or 30 mg/kg VU0467154 compared to 30 mg/kg VU0467154 with 10 mg/kg VU6028418 (RM 1-way ANOVA followed by Dunnett's (D-F) or Sidak's (J-L) test), n=14/group; See table 5.6 for full statistical analysis.

5.3F).

During the active phase 30 mg/kg VU0467154 again produced reduced wake and increased NREM sleep. VU6028418 reversed the observed reduction in wake when dosed with VU0467154 and produced increased wake when dosed alone when assessed in 2-hour bins (Figure 5.3G) or 12-hour bins (Figure 5.3J). The increased time in NREM sleep observed following VU0467154 dosing in the active phase is similarly blocked by VU6028418 assessing in both 2-hour and 12-hour bins. Effects of the M_4 mAChR PAM on REM sleep when dosed during the active phase are minimal with a decrease seen at ZT18, and a decrease in the subsequent inactive phase. The M_4 mAChR antagonist VU6028418 reverses the reductions in REM sleep seen in the active phase subsequent to dosing when assessed in 2-hour bins (Figure 5.3I) or 12-hour bins (Figure 5.3L).

The M_4 mAChR PAM VU0467154 increases NREM sleep bout duration in young mice when dosed in the active phase, which is attenuated by the M_4 mAChR antagonist VU6028418.

In young mice, VU0467154 dosing produced a main effect on NREM sleep bout number and NREM sleep bout duration. 1mg/kg VU0467154 increased NREM sleep bout number (Figure 5.4A), with 10 and 30 mg/kg increasing NREM sleep bout duration (Figure 5.4B).

During the active phase in combination studies 30 mg/kg VU0467154 produced no effect on NREM sleep bout number compared to vehicle, while combination dosing with the M_4 mAChR

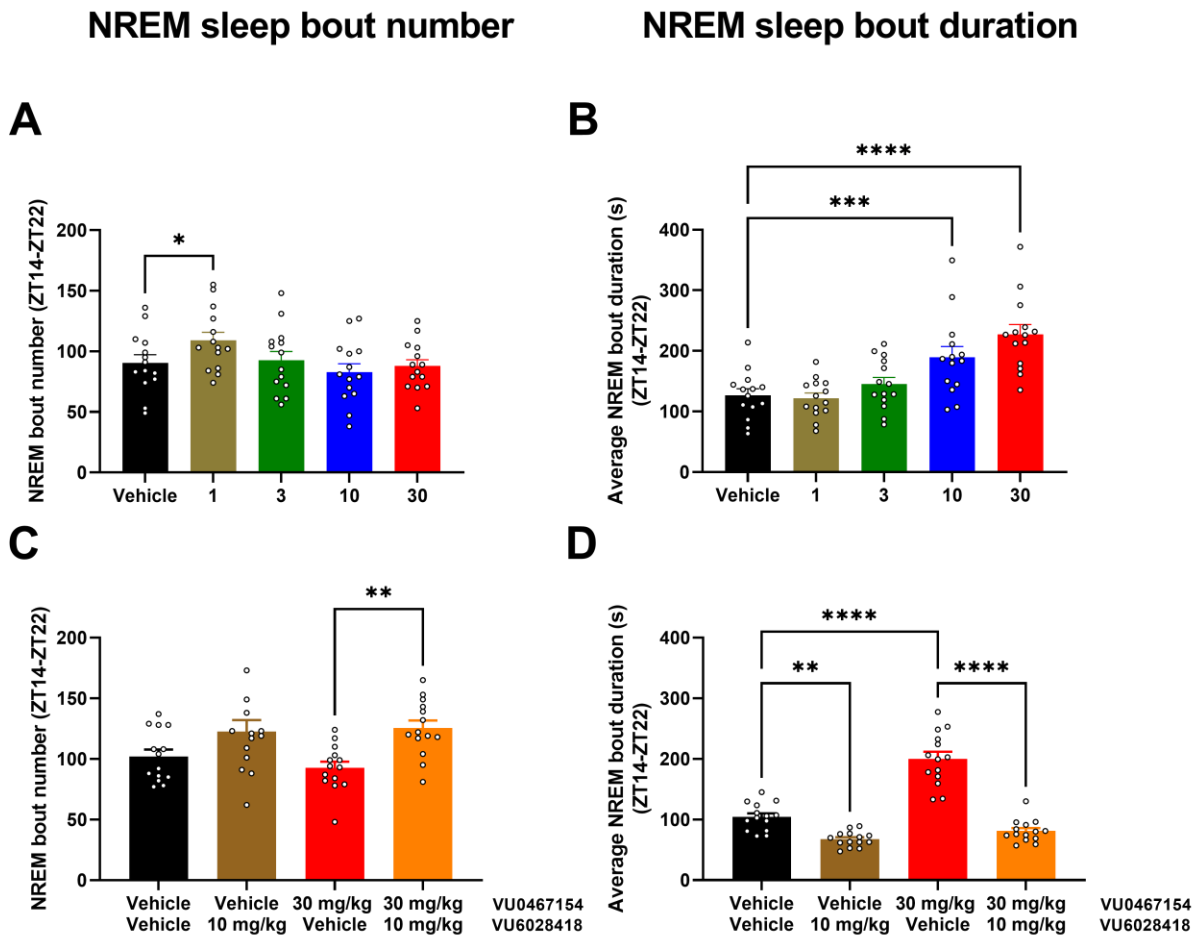

Figure 5.4. The M_4 mAChR PAM VU0467154 increased NREM sleep bout duration during the active phase in young mice, which was attenuated by VU6028418. Shown is the average NREM sleep bout number (A, C) and the average NREM sleep bout duration (B, D) in young mice for 8 hours following dosing in the active phase (ZT14-ZT22). 1 mg/kg VU0467154 increased NREM sleep bout number in young mice (A) and 10 and 30 mg/kg increased NREM sleep bout duration (B). VU6028418 dosed with VU0467154 increased NREM sleep bout number compared to 30 mg/kg VU0467154 (C), and 10 mg/kg VU6028418 reversed the observed increase in NREM sleep bout duration following 30 mg/kg VU467154 (D). Data are expressed as overall means ± S.E.M., n=14/group. * indicates p<0.05, **p<0.01, *** p<0.001 and **** p<0.0001 compared to vehicle, or 30 mg/kg VU0467154 compared to 30 mg/kg VU0467154 with 10 mg/kg VU6028418 (RM 1-way ANOVA followed by Dunnett's (A-C) or Sidak's (D-F) test). See table 5.6 for full statistical analysis.

antagonist 10 mg/kg VU6028418 produced a significant increase compared to 30 mg/kg VU0467154 alone (Figure 5.4C). During the active phase 30 mg/kg VU0467154 increased NREM sleep bout duration, which was reversed when dosed in combination with the M_4 mAChR

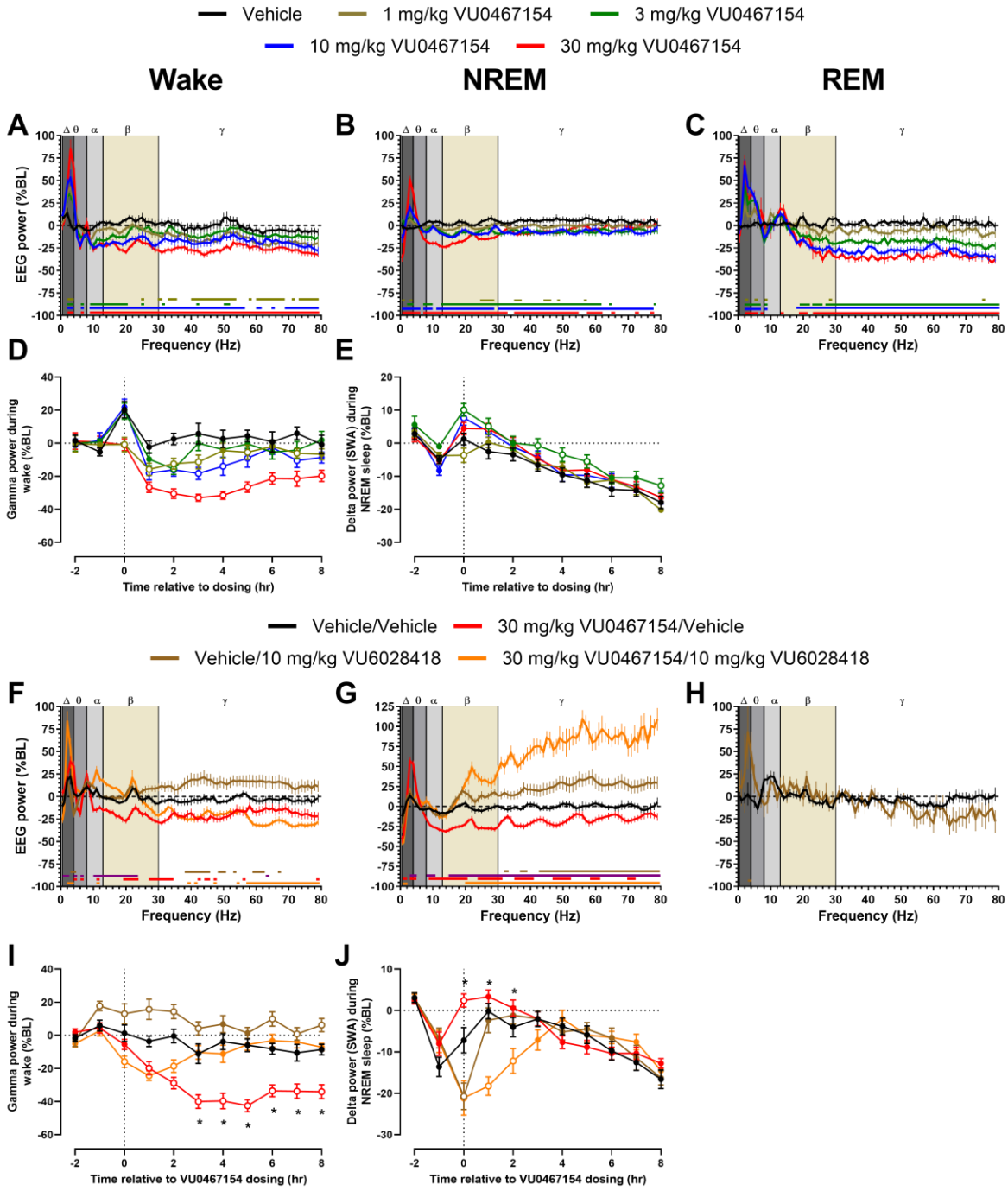

Figure 5.5. The M_4 mAChR PAM VU0467154 produced dose dependent shifts to lower frequencies during all sleep/wake states during the inactive phase in young mice, which was attenuated by VU6028418. Shown is the relative spectral power during wake (A, F), NREM sleep (B, G) and REM sleep (C, H) epochs only in the 1-2 hours following VU0467154 (VU0467154 administration 2 hours following light change (A-E), VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change for combination studies (F-J)) dosing relative to the 1-hour predose baseline during the inactive phase, and relative gamma power during wake (D, I) and relative delta power (SWA) during NREM sleep (E, J), across the inactive phase in young (A-E) and non-pathologically aged (F-J) mice. In young mice, VU0467154 produced shifts to lower frequencies at all doses tested during wake (A), NREM sleep (B) and REM sleep (C). VU0467154 dose dependently reduced gamma power during wake (D) and transiently increased relative delta power (SWA) during NREM sleep (E). VU0467154 30 mg/kg produced shifts to lower frequencies during wake (F) and NREM sleep (G) during the inactive phase, resulting in decreased gamma power during wake (F) and transiently increased delta power during NREM sleep (G). Insufficient mice displayed REM sleep in the 1-2 hours following VU0467154 dosing for analysis (H). 10 mg/kg VU6028418 increased gamma power during wake in the inactive phase (F, I) and produced a shift to higher frequencies during NREM sleep producing a transient decrease in delta power (SWA) (G, J). No effect was observed on REM sleep (H). 30 mg/kg VU0467154 dosed in combination with 10 mg/kg VU6028418 produced a shift to lower frequencies during wake in the 1-2 hours following dosing (F), however blockade of VU0467154 induced decreased gamma power was observed from 3 hours following dosing (I). During NREM sleep 30 mg/kg VU0467154 dosed in combination with 10 mg/kg VU6028418 produced increased gamma power and decreased delta power (SWA) (G, J). Insufficient mice displayed REM sleep in the 1-2 hours following VU0467154 dosing in combination with 10 mg/kg VU6028418 for analysis (H). Data are expressed as means ± S.E.M. in 1Hz bins (A-C, F-H) and means in 1hr bins ± S.E.M. (D, E, I, J), n=14/group. Solid bars and open symbols indicate $p<0.05$ compared to vehicle, purple solid bars and * indicates 30 mg/kg VU0467154 $p<0.05$ compared to VU0467154 30 mg/kg dosed with 10 mg/kg VU6028418 (RM 2-way ANOVA matching by both factors followed by Dunnett's (A-E) or Sidak's (F-J) test). See table 5.6 for full statistical analysis.

antagonist VU6028418. 10 mg/kg VU06028418 dosed alone produced a reduction in NREM sleep bout duration (Figure 5.4D).

When assessing wake bout duration in the active phase following VU0467154 dosing, young mice displayed a main effect of dose on wake bout number, but no effect at any individual dose on post hoc comparisons. Young mice display significantly reduced wake bout duration at 1 and 30 mg/kg (Table 5.1).

During the active phase in combination dosing studies 30 mg/kg VU0467154 produced no effect on wake bout number compared to vehicle following, while combination dosing with 10 mg/kg of the M_4 mAChR antagonist VU6028418 produced a significant increase compared to 30 mg/kg VU0467154 alone. VU0467154 and VU6028418 combination dosing during the active produced a main effect of dose on wake bout duration, with no effect seen at any individual dose following post hoc assessments (Table 5.1).

The M_4 mAChR PAM VU0467154 produces shifts to lower powers in all arousal states across phase in young mice, which is attenuated by the M_4 mAChR antagonist VU6028418.

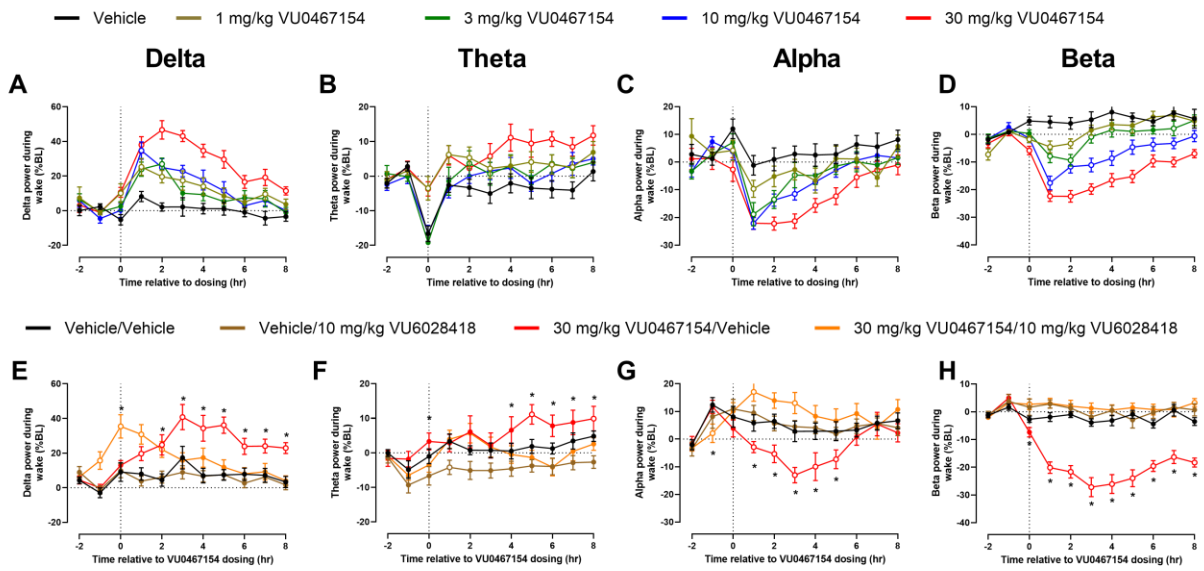

Figure 5.6. The M_4 mAChR PAM VU0467154 produced a dose dependent shift to lower frequency power bands during wake during the inactive phase in young mice, which is attenuated by the M_4 mAChR antagonist VU6028418. Shown is the change in relative spectral within the delta (A, E), theta (B, F), alpha (C,G) and beta (B,H) powerbands during wake in young mice for 8 hours following VU0467154 dosing 2 hours into the inactive phase (A-D) or VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change (E-H). VU0467154 dose dependently increased delta power in young mice (A) which was attenuated by VU6028418 (E). Theta power was increased dose dependently following VU0467154 dosing in young mice (C) which was attenuated by VU6028418 (F). VU0467154 dose dependently decreased alpha power in young mice (E) which was attenuated by VU6028418 (G). VU0467154 dose dependently decreased beta power in young mice (G) which was attenuated by VU6028418 (H) mice. Data are expressed as means in 1hr bins ± S.E.M., n=14/group. Open symbols indicate $p<0.05$ compared to vehicle, and * indicates 30 mg/kg VU0467154/vehicle $p<0.05$ compared to 30 mg/kg VU0467154/10 mg/kg VU6028418 (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 5.6 for full statistical analysis.

When assessing spectral power during wake, VU0467154 dosed in the inactive phase in young mice produced a shift to lower frequency powers. All doses increased delta power, with 3, 10 and 30 mg/kg displaying reductions in theta, alpha and beta power. 1, 10 and 30 mg/kg displayed reductions in gamma power (Figure 5.5A), resulting in decreased gamma power across the inactive phase with dose-dependent reductions observed following dosing (Figure 5.5D). Consistent with this shift to lower frequencies reduced alpha and beta frequencies with increased delta and theta frequencies are seen across the inactive phase (Figure 5.6). In young mice dosed with VU0467154 in the inactive period, there was a dose-related shift from higher to lower frequencies during NREM sleep. Most pronounced effects were seen at 10 and 30 mg/kg with decreases in low delta, increases in higher delta and decreases throughout theta, alpha, beta,

and gamma ranges (Figure 5.5B). Following VU0467154 dosing increased delta power (slow wave activity (SWA)) during NREM sleep is observed, with a modest increase seen at 3, 10 and 30 mg/kg (Figure 5.5E). Consistent with this shift to lower frequencies increased theta, and reduced alpha, beta and gamma frequencies are observed (Figure 5.7). During REM sleep in young mice all doses tested increased delta and theta frequencies, with 3, 10 and 30 mg/kg decreasing alpha and gamma frequencies (Figure 5.5C).

Similar to previous results, following dosing in the inactive phase 30 mg/kg of the M_4 mAChR PAM VU0467154 produced a shift to lower powers during wake as seen by a reduction in alpha, beta and gamma powers (Figure 5.5F), with a decrease in gamma power across the inactive phase following dosing (Figure 5.5I). Combination dosing with the M_4 mAChR antagonist

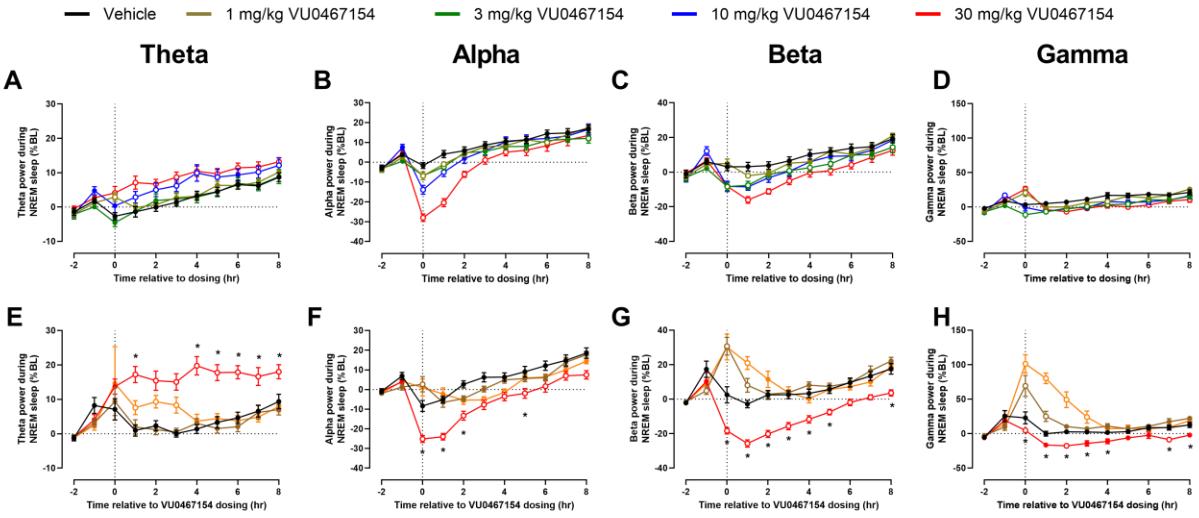

Figure 5.7. The M_4 mAChR PAM VU0467154 produced a dose dependent shift to lower frequency power bands during NREM sleep in the inactive phase in young and non-pathologically aged mice. Shown is the change in relative spectral within the theta (A, E), alpha (B, F), beta (C, G) and gamma (B, H) powerbands during wake in young mice for 8 hours following VU0467154 dosing 2 hours into the inactive phase (A-D) or VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change (E-H). Theta power was increased following VU0467154 dosing at 10 and 30 mg/kg in young mice (A) which was attenuated by VU6028418 (E). VU0467154 dose dependently decreased alpha power in young mice (B) which was attenuated by VU6028418 (F). VU0467154 dose-dependently decreased beta power in young mice (C) which was attenuated by VU6028418 (G) mice. VU0467154 dose dependently decreased gamma power in young mice, following a transient increase following 1 and 30 mg/kg dosing (D) while dosing in combination with VU6028418 produced a robust increase in gamma power (H). Data are expressed as means in 1hr bins ± S.E.M., n=14/group. Open symbols indicate $p<0.05$ compared to vehicle, and * indicates 30 mg/kg VU0467154/vehicle $p<0.05$ compared to 30 mg/kg VU0467154/10 mg/kg VU6028418 (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 5.6 for full statistical analysis.

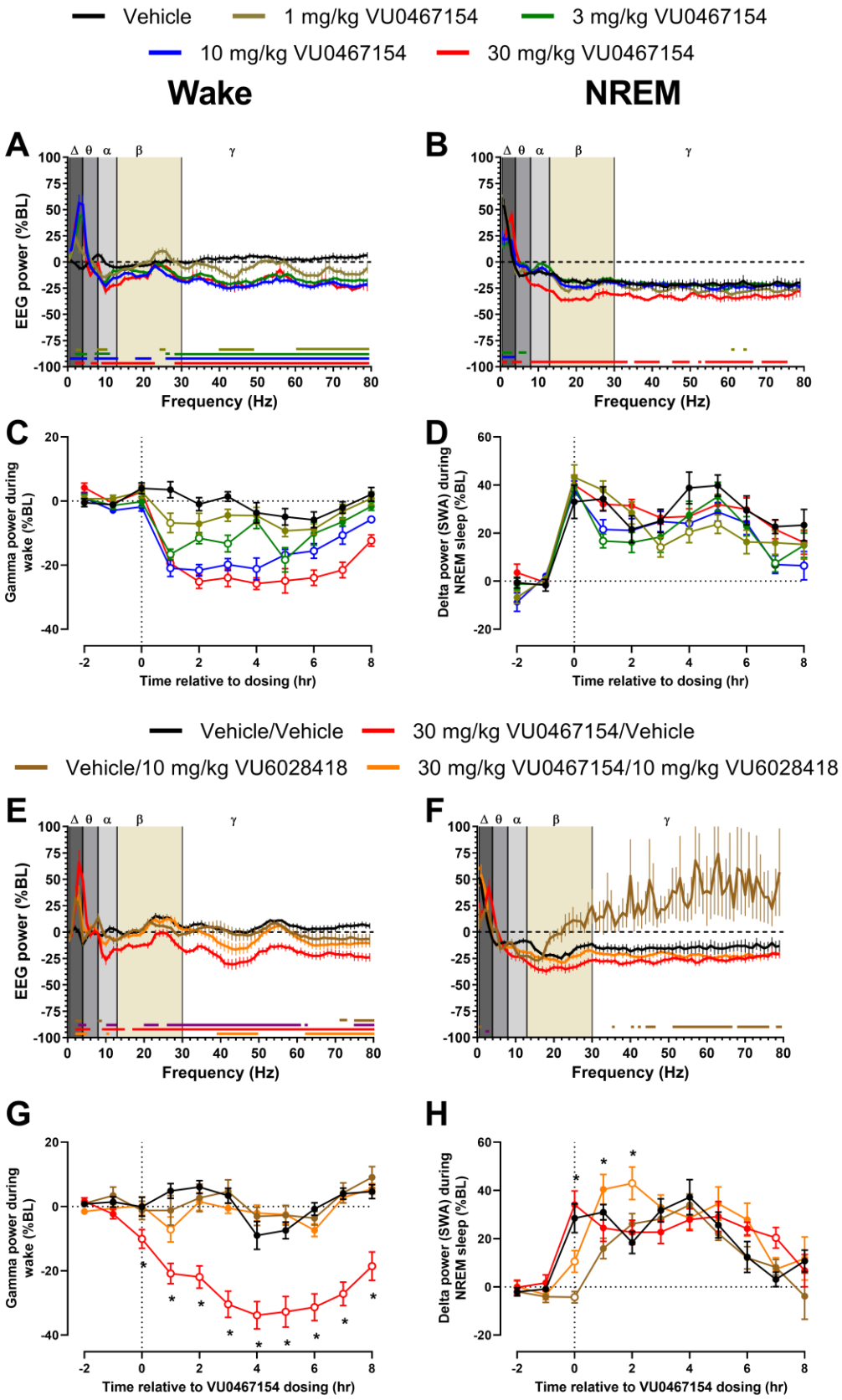
Vehicle
1 mg/kg VU0467154
3 mg/kg VU0467154
10 mg/kg VU0467154
30 mg/kg VU0467154
Wake
NREM
A
B
C
D
Δ θ α β γ
EEG power (%BL)
Frequency (Hz)
Gamma power during wake (%BL)
Delta power (SWA) during NREM sleep (%BL)
Time relative to dosing (hr)
Vehicle/Vehicle
30 mg/kg VU0467154/Vehicle
Vehicle/10 mg/kg VU6028418
30 mg/kg VU0467154/10 mg/kg VU6028418
E
F
G
H
Time relative to VU0467154 dosing (hr)

Figure 5.8. The M_4 mAChR PAM VU0467154 produced shifts to lower powers during wake and NREM sleep in the active phase of young mice, which during wake was attenuated by VU6028418. Shown is the relative spectral power during wake (A, E) and NREM sleep (B, F) epochs only in the 1-2 hours following VU046154 dosing relative to the 1-hour predose baseline during the active phase, and gamma power during wake (C, G) and relative delta power (SWA) during NREM sleep (D, H), across the active phase in young (A-D) and non-pathologically aged (E-H) mice. In young mice, VU0467154 produced shifts to lower frequencies at all doses tested during wake (A), and at 30 mg/kg during NREM sleep (B). VU0467154 dose-dependently reduced gamma power during wake (C) and decreased relative delta power (SWA) during NREM sleep at 1, 3 and 10 mg/kg (D). During the active phase 30 mg/kg VU0467154 produced a shift to lower frequencies during wake (E), producing reduced gamma power (G). No effect of 30 mg/kg VU0467154 was observed on NREM sleep (F, H). 10 mg/kg VU6028418 produced no effect qEEG during wake (E, G), however produced a shift to higher frequencies during NREM sleep producing a transient decrease in delta power (SWA) following dosing (F, H). 30 mg/kg VU0467154 dosed with 10 mg/kg VU6028418 blocked the shift to lower frequencies and decreased gamma during wake observed with VU0467154 alone (E, G). During NREM sleep VU0467154 30 mg/kg dosed with 10 mg/kg VU6028418 produced no effect on qEEG 1-2 hours following dosing (F), however a transient decrease, and then increase in delta power (SWA) was observed which was significantly different to 30 mg/kg VU0467154 alone 0-2 hours after dosing (H). Data are expressed as means ± S.E.M. in 1Hz bins (A-B, E-F) and means in 1hr bins ± S.E.M. (C, D, G, H), n=14/group. Solid bars and open symbols indicate $p<0.05$ compared to vehicle, purple solid bars and * indicates 30 mg/kg VU0467154 $p<0.05$ compared to VU0467154 30 mg/kg dosed with 10 mg/kg VU6028418 (RM 2-way ANOVA matching by both factors followed by Dunnett's (A-D) or Sidak's (E-H) test). See table 5.6.

VU6028418 at 10 mg/kg reverses VU0467154 effects in the alpha and beta power bands but not gamma 1-hour post-dosing. When dosed alone VU6028418 increased gamma power (Figure 5.5F). Over the inactive phase the VU0467154 dependent reduction in gamma power is reversed following combination dosing with 10 mg/kg VU6028418 from 3-hrs after dosing. 10 mg/kg VU6028418 alone increased gamma power across the inactive phase (Figure 5.5I). Consistent with this, the VU0467154 dependent shift to lower frequencies observed in the inactive phase is blocked by VU6028418 during the inactive phase (Figure 5.6). During NREM sleep, when dosed in the inactive phase, 30 mg/kg VU0467154 produced decreased alpha, beta and gamma with increased high delta and decreased low delta observed. 10 mg/kg VU6028418 increased gamma power during NREM sleep, while combination dosing with 30 mg/kg VU0467154 resulted in a greater increase in gamma power, increased beta power and decreased delta power (Figure 5.5G). These effects are consistent with the effects seen in delta power (SWA) across the inactive phase, where 30 mg/kg VU0467154 produced a transient increase, 10 mg/kg VU6028418 produced a transient decrease and the combination of the 30 mg/kg VU0467154 and 10 mg/kg VU6028418 produced a more robust decrease (Figure 5.5J). During NREM sleep 30 mg/kg VU0467154 increased theta and decreased alpha and beta powers across the inactive period,

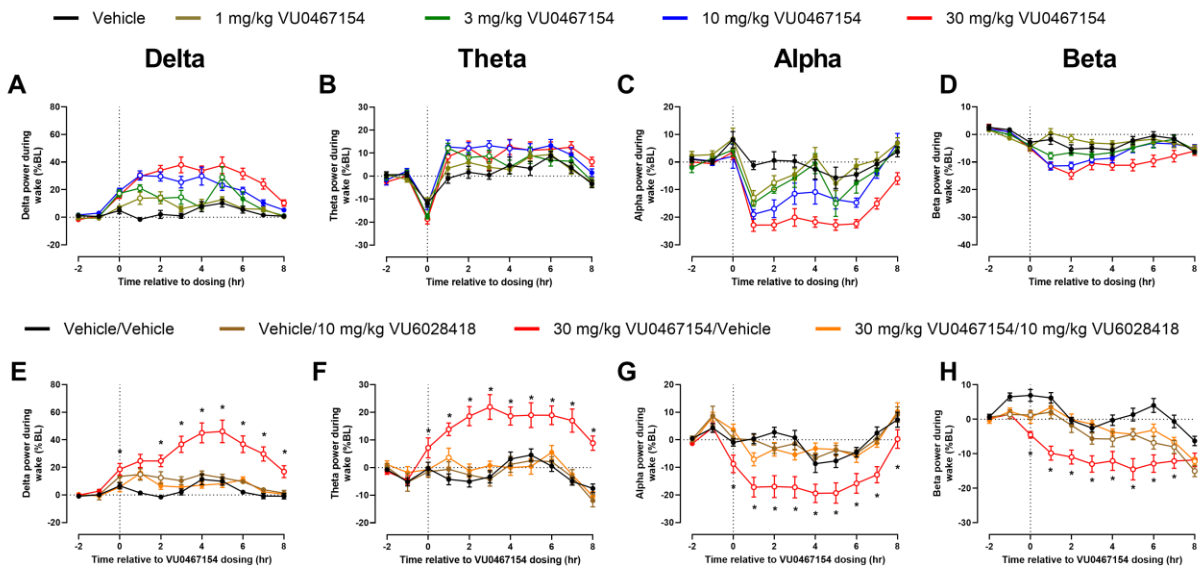

Figure 5.9. The M_4 mAChR PAM VU0467154 produced a dose dependent shift to lower frequency power bands during wake in the active phase in young mice, which was attenuated by the M_4 mAChR VU6028418. Shown is the change in relative spectral within delta (A, E), theta (B, F), alpha (C,G) and beta (B,H) powerbands during wake in young mice for 8 hours following VU0467154 dosing 2 hours into the active phase (A-D) or VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change (E-H). VU0467154 dose dependently increased delta power in young mice (A) which was attenuated by VU6028418 (E). Theta power was increased following VU0467154 dosing at 3, 10 and 30 mg/kg in young mice (B) which was attenuated by VU6028418 (F). VU0467154 dose dependently decreased alpha power in young mice (C) which was attenuated by VU6028418 (G). VU0467154 dose dependently decreased beta power in young mice (D) which was attenuated by VU6028418 (H). Data are expressed as means in 1hr bins ± S.E.M., n=14/group. Open symbols indicate p<0.05 compared to vehicle, and * indicates 30 mg/kg VU0467154/vehicle p<0.05 compared to 30 mg/kg VU0467154/10 mg/kg VU6028418 (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 5.6 for full statistical analysis.

which were reversed by 10 mg/kg VU6028418 (Figure 5.7). During REM sleep in the inactive phase 10 mg/kg VU6028418 produced an increase in relative power at 3Hz (Figure 5.5H). In both the 30 mg/kg VU0467154 alone and in combination with 10 mg/kg VU6028418 groups fewer than five mice displayed REM sleep in the 1-2 hours following dosing, so no data is shown.

When dosed with VU0467154 in the active phase young mice displayed a shift in relative spectral power to lower frequencies during wake epochs. All doses increased delta power, decreased alpha and gamma, with 10 and 30 mg/kg displaying reductions in beta power (Figure 5.8A), resulting in a dose-dependent decrease in gamma power during wake across the phase following dosing (Figure 5.8C). Consistent with this shift to lower frequencies increased delta and

theta frequencies, with decreased alpha and beta frequencies are observed (Figure 5.9). During NREM sleep epochs VU0467154 produced a reduction in gamma, beta and alpha frequencies at 30 mg/kg, and a reduction in delta power at 3 and 10 mg/kg (Figure 5.8B). VU0467154 at 1, 3 and 10 mg/kg produced modest reductions in delta power (SWA) during NREM sleep (Figure 5.8D). Consistent with this shift to lower frequencies VU0467154 produced decreased alpha, beta and frequencies during NREM sleep (Figure 5.10).

During the active phase 30 mg/kg VU0467154 produces a shift to lower frequencies during wake as previously described, resulting in increased delta power, with reduced alpha, beta and gamma powers. These effects are reversed following combination dosing with 10 mg/kg of the M_4 mAChR antagonist VU6028418. When dosed alone VU6028418 produced modest reductions in

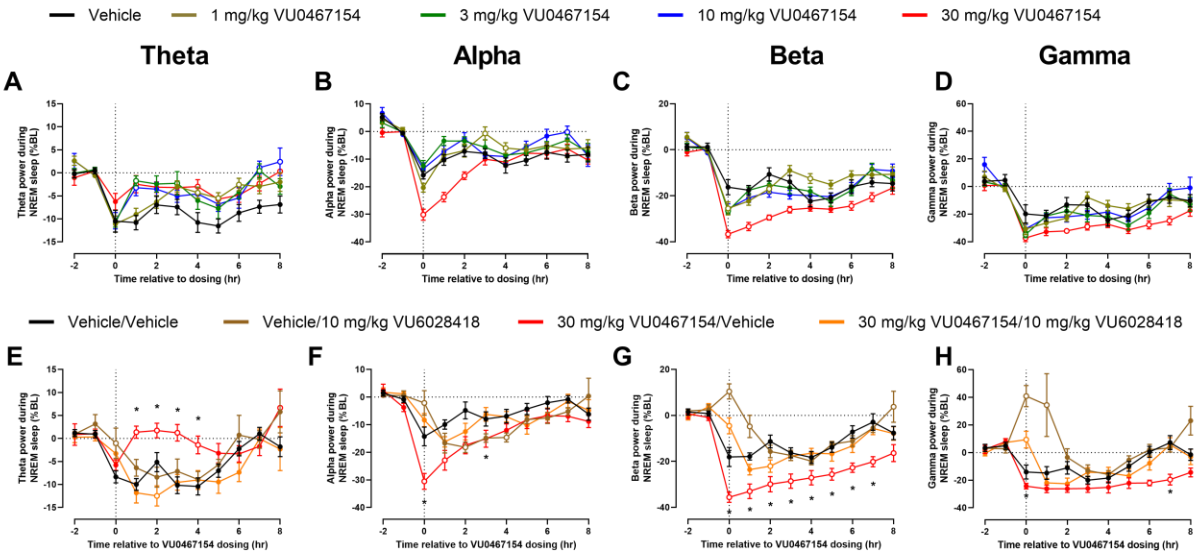

Figure 5.10. The M_4 mAChR PAM VU0467154 produced a dose dependent shift to lower frequency power bands during NREM sleep in the active phase in young mice, which was attenuated by the M_4 mAChR VU6028418. Shown is the change in relative spectral within the theta (A, E), alpha (B, F), beta (C, G) and gamma (B, H) powerbands during wake in young mice for 8 hours following VU0467154 dosing 2 hours into the active phase (A-D) or VU6028418 administration 2 hours following light change and VU0467154 administration 2.5 hours following light change (E-H). Theta power was increased following VU0467154 dosing at all doses in young mice (A) which was attenuated by VU6028418 (E). VU0467154 decreased alpha power at 30 mg/kg in young (B) which was attenuated by VU6028418 (F). VU0467154 dose dependently decreased beta power in young mice (C) which was attenuated by VU6028418 (F). 30 mg/kg VU0467154 decreased gamma power in young mice (D) which was attenuated by VU6028418 (H). Data are expressed as means in 1hr bins ± S.E.M., n=14/group. Open symbols indicate $p<0.05$ compared to vehicle, and * indicates 30 mg/kg VU0467154/vehicle $p<0.05$ compared to 30 mg/kg VU0467154/10 mg/kg VU6028418 (RM 2-way ANOVA matching by both factors followed by Dunnett's or Sidak's test). See table 5.6 for full statistical analysis.

high gamma power and reduced delta power (Figure 5.8E). When assessing gamma power across the active phase a robust reduction in gamma power is observed following 30 mg/kg VU0467154 dosing which is completely reversed by 10 mg/kg VU6028418 (Figure 5.8G). These shifts to lower powers being blocked by the M_4 antagonist VU6028418 are consistent with the effect seen in delta, theta, alpha and beta power bands during wake across the active phase (Figure 5.9). During NREM sleep in the active phase no effect on qEEG was observed following dosing with 30 mg/kg VU0467154. VU6028418 dosed alone increased gamma power during NREM sleep in the active phase (Figure 5.8F). 30 mg/kg VU0467154 produced no effect on delta power (SWA) during NREM sleep across the active phase, 10 mg/kg VU6028418 alone and in combination with 30 mg/kg VU0467154 produced a transient decreased delta power (SWA) during NREM sleep, with a transient rebound increase observed in the combination dosing group

	Young (SEM)	**Aged (SEM)**	**t-test**	**P value**
Wake				
Inactive (ZT2-ZT10)				
Wake Bout Number	113.7 (5.95)	142.2 (16.5)	t, df=1.627, 24	0.1167
Average Wake Bout Duration (s)	73.70 (3.20)	69.56 (6.44)	t, df=0.5744, 24	0.5711
Active (ZT14-ZT22)				
Wake Bout Number	95.38 (6.95)	128.2 (5.86)	t, df=3.611, 24	0.0014
Average Wake Bout Duration (s)	198.1 (20.5)	143.1 (11.4)	t, df=2.351, 24	0.0273
NREM sleep				
Inactive (ZT14-ZT22)				
NREM Bout Number	113.2 (6.49)	131.2 (9.96)	t, df=1.521, 24	0.1414
Average NREM Bout Duration (s)	170.2 (12.7)	144.5 (9.77)	t, df=1.672, 24	0.1076
Active (ZT14-ZT22)				
NREM Bout Number	90.62 (7.18)	124.2 (6.06)	t, df=3.577, 24	0.0015
Average NREM Bout Duration (s)	128.4 (11.3)	86.72 (7.31)	t, df=3.095, 24	0.0049

Table 5.2. Non-pathologically aged mice display increased fragmentation of wake and NREM sleep during the active phase. Shown is the average wake bout number and average wake duration during the inactive and active phase for the 8-hours following vehicle dosing in young and non-pathologically aged mice. N=13 per group, unpaired t-test performed comparing young to aged.

(Figure 5.8H). VU0467154 produced increased theta power, reduced alpha, beta and gamma power during NREM sleep in the active period, all of which were reversed by VU6028418 (Figure 5.10).

Non-pathologically aged mice displayed reduced REM sleep during the inactive phase and increased sleep/wake fragmentation during the active phase when compared to young mice.

Non-pathologically aged mice displayed significant reductions in REM sleep during the inactive phase. No age-related change wake or NREM sleep were observed following posthoc analysis (Figure 5.11). When assessed across the 12 hours of the inactive and active periods aged mice displayed no change in wake on post-hoc assessments or NREM sleep, but a significant reduction in REM during the inactive phase (Figure 5.11D-F). Nonpathologically aged mice displayed no change in NREM sleep or wake bout number, or NREM sleep or wake bout duration during the inactive phase. During the active phase aged animals display a significant

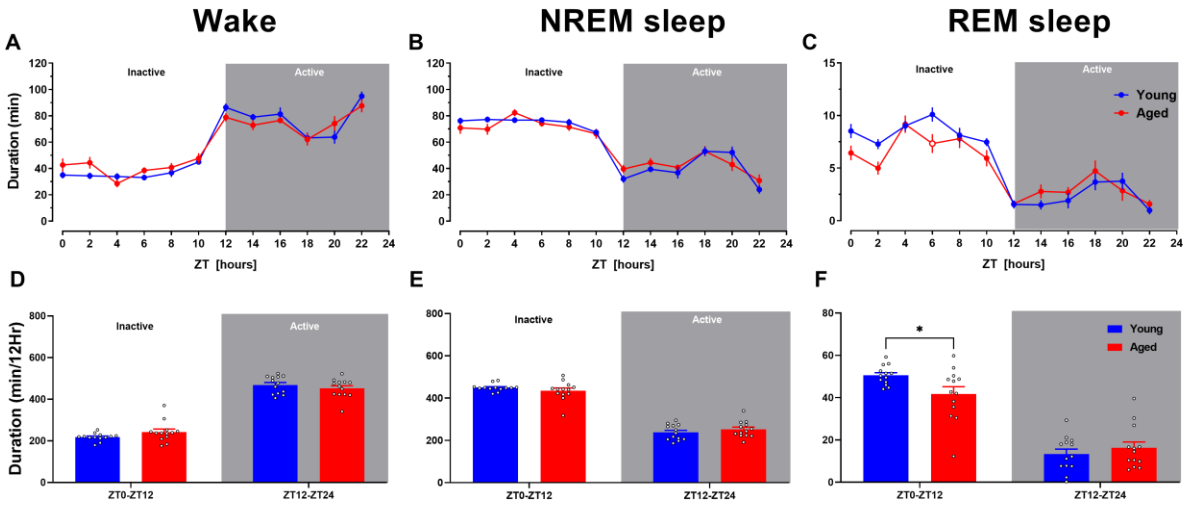

Figure 5.11. Non-pathologically aged mice displayed reduced REM sleep during the inactive phase when compared to young mice. Shown is the duration of time in wake (A, D), NREM sleep (B, E) and REM sleep (C, F) in young (4-5-month) and non-pathologically aged (20-21-month) mice. No age-related change in wake (A, D) or NREM sleep were seen (B, E). Aged mice displayed a significant decrease in REM sleep between ZT0-ZT12 (C-F). Data are expressed as means ± S.E.M. of 2-hour bins (A-C); total duration of time in minutes in wake, NREM sleep and REM sleep respectively ± S.E.M in 12hrs bins (D-F); n=13/group; open circles indicate $p<0.05$ (C), ** indicate $p<0.01$ (F) compared to young (RM 2-way ANOVA matching by time followed by Sidak's test). See table 5.6 for full statistical analysis.

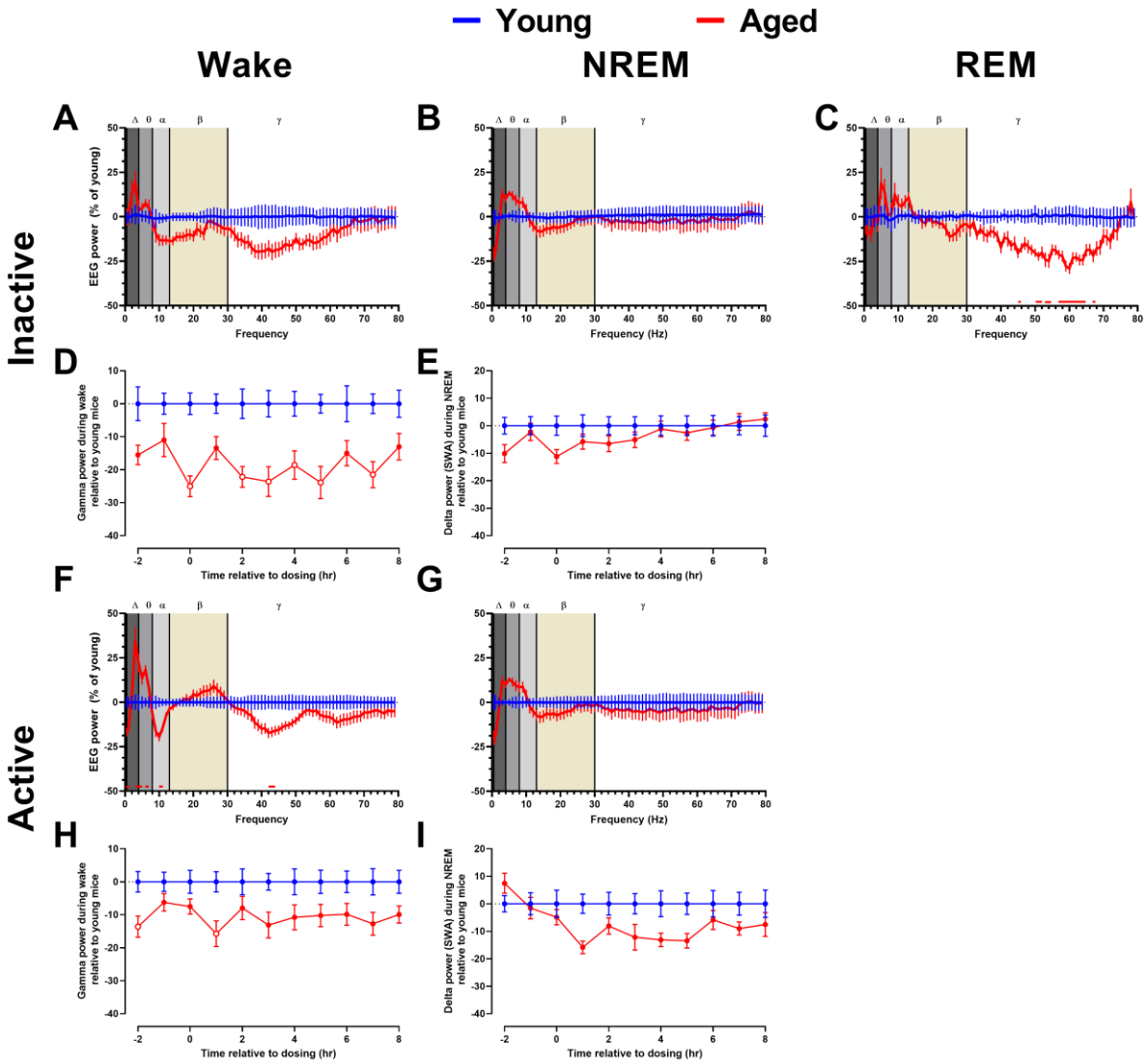

Figure 5.12. Non-pathologically aged mice displayed reduced arousal in both phases. Shown is the relative spectral power in non-pathologically aged (20-21-month) mice normalized to young (4-5-month) mice from 0.5-80Hz during wake (A, F), NREM sleep (B, G), and REM sleep (C) during ZT1-2 (vehicle baseline) in the inactive phase (A-C) and the active phase (F, G). The relative gamma power during wake normalized to young mice (D, H) and the relative delta power (SWA) during NREM sleep (E, I) normalized to young mice in the inactive phase (D, E) and the active phase (H, I). During the inactive phase young and aged mice displayed a significant frequency x age interaction during wake with a relative shift to lower frequencies (A), with a significant reduction in gamma power (D). During NREM sleep a significant frequency x age interaction was observed (B), with no change in relative delta power (SWA) during NREM sleep (E). During REM sleep a main effect of age was observed with shifts from gamma to theta and alpha frequencies (C). During the active phase young and aged mice displayed a significant frequency x age interaction during wake with a relative shift to lower frequencies (F), with a reduction in gamma power seen in aged mice (H). During NREM sleep no significant change is seen (G), with a no change in SWA (relative delta power) during NREM sleep (I). Data are expressed as means ± S.E.M. in 1Hz bins (A-C, F, G) and mean in 1hr bins ± S.E.M. (D, E, H, I), n=13/group. Solid bars or open symbols indicate $p<0.05$ compared to young (RM 2-way ANOVA matching by time followed by Sidak's test). See table 5.6 for full statistical analysis.

increase in NREM sleep bout number and a significant decrease in NREM sleep bout duration (Table 5.2). Wake bouts during the active phase displayed significant age-related increases in bout number and average duration (Table 5.2).

Non-pathologically aged mice display reduced arousal during wake epochs across phase.

During the inactive phase, spectral power during wake displayed no changes at any frequency on post hoc tests during the baseline period, although an age x frequency interaction was observed. A visual trend towards a shift in lower powers was observed but this did not reach significance at any frequency (Figure 5.12). When assessing gamma power during wake epochs, a correlate of arousal, an age-related decrease was observed (Figure 5.12D). In contrast, during the active phase during wake epochs there was significantly increased delta and theta powers in aged mice, with reductions in alpha and gamma frequencies (Figure 5.12F). Reduced gamma power was also observed during wake epochs in the active phase in non-pathologically aged mice (Figure 5.12H).

During the inactive phase NREM sleep qEEG showed no change in spectral power at any frequency following post hoc tests, although an age x frequency interaction was observed (Figure 5.12B). No change in delta frequency (SWA) during NREM sleep in non-pathologically aged mice following post hoc tests in the inactive phase, although an age x time interaction was observed (Figure 5.12E). In the active phase no significant age-related changes were seen on spectral power at any frequency following post hoc analysis during NREM sleep was observed, although an age x frequency interaction was seen (Figure 5.12G). Delta power (SWA) during NREM sleep displayed a main effect of age and an age x frequency interaction, although this did not reach significance at any time on post hoc tests (Figure 5.12I). REM sleep during the inactive phase displayed age-related increased theta power and reduced gamma power (Figure 5.12C).

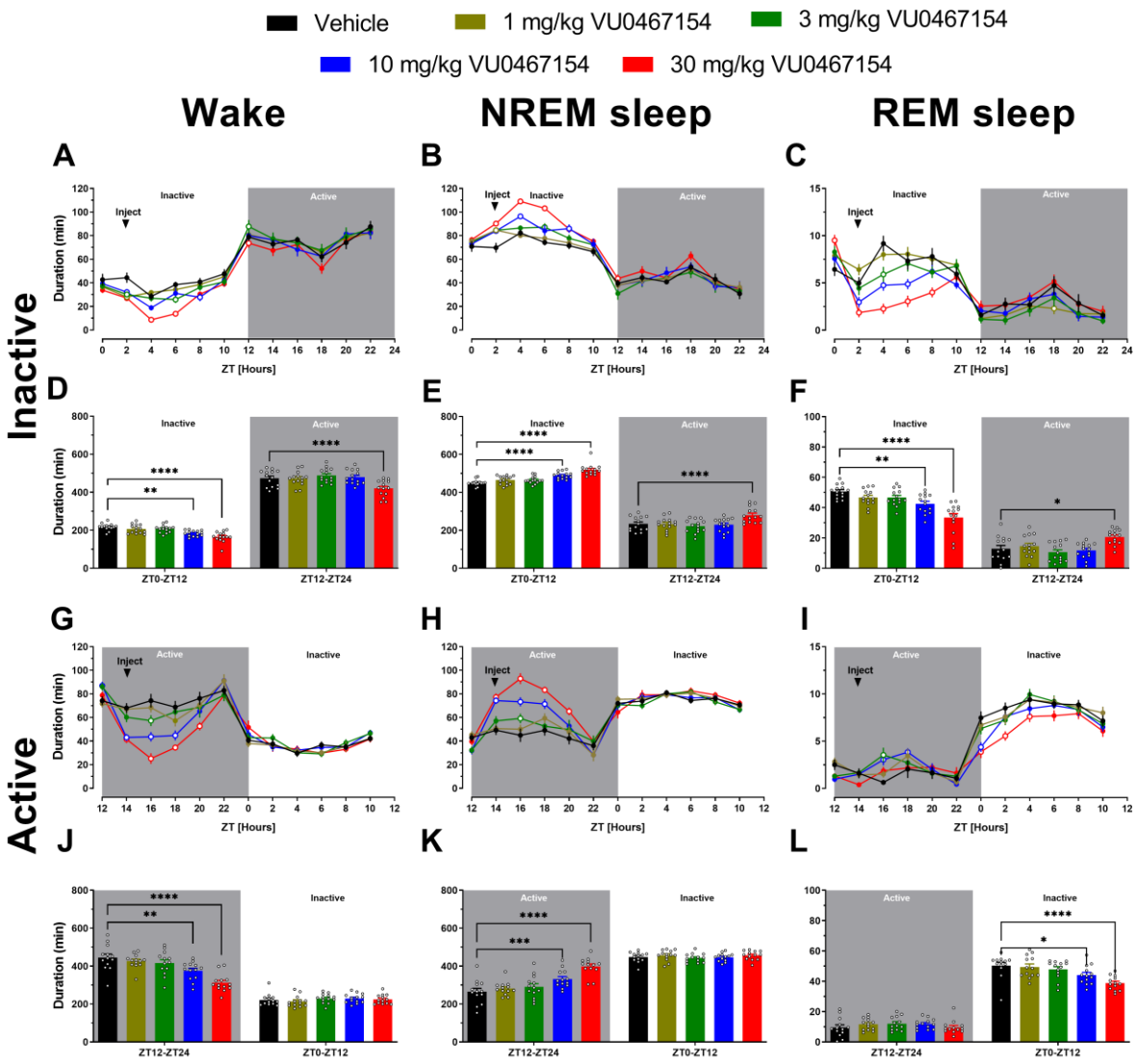

Figure 5.13. The M_4 mAChR PAM VU0467154 produced dose dependent increases in NREM sleep and reduced REM sleep during the inactive and active phase in non-pathologically mice. Shown is the duration of time in wake (A, D), NREM sleep, (B, E) and REM sleep (C, F) in non-pathologically aged (20-21-month) mice following VU0467154 administration 2 hours into the inactive period (see arrowhead). During the inactive phase 1, 3, 10 and 30 mg/kg VU0467154 decreased duration of time in wake following dosing (A), with decreased wake seen across the 12 hours of the inactive phase at 10 and 30 mg/kg (D). 1, 3, 10 and 30 mg/kg VU0467154 increased duration of time in NREM sleep following dosing (B), with increased NREM sleep seen across the 12 hours of the inactive phase at 10 and 30 mg/kg (E). 3, 10 and 30 mg/kg VU0467154 reduced duration of time in REM sleep following dosing with an increase in duration of time in REM sleep also seen at baseline in the 30 mg/kg group (C) with decreased REM sleep seen across the 12 hours of the inactive phase at 10 and 30 mg/kg (F). During the active phase 3, 10 and 30 mg/kg VU0467154 decreased duration of time in wake following dosing (G), with reduced wake seen across the 12 hours of the active phase following 10 and 30 mg/kg dosing (J). 3, 10 and 30 mg/kg VU0467154 increased duration of time in NREM sleep following dosing (H), with increased NREM sleep seen across the 12 hours of the active period at 10 and 30 mg/kg (K). 3 mg/kg and 10 mg/kg VU0467154 increased duration of time in REM sleep following dosing with 10 and 30 mg/kg reducing REM sleep into the inactive phase (I), across the 12 hours of the active phase, no dose alters REM sleep duration, with 10 and 30 mg/kg reducing REM sleep into the subsequent inactive phase (L). Data are expressed as means ± S.E.M. of 2-hour bins (A-C, G-I) open symbols indicate $p<0.05$ compared to vehicle (2-way ANOVA matching by both factors followed by Dunnett's test), or 12-hour bins (D-F, J-L) * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ compared to vehicle (RM 1-way ANOVA followed by Dunnett's test), n=13/group; See table 5.6 for full statistical analysis.

In non-pathologically aged mice VU0467154 increased NREM sleep across phase and reduced REM sleep during the inactive phase.

In non-pathologically aged mice when dosed in the inactive period, VU0467154 reduced time in wake. A transient reduction in wake was seen at 1 and 3 mg/kg with a more prolonged wake reduction seen at 10 and 30 mg/kg (Figure 5.13A), when assessed across the 12 hours of the inactive and active phases a reduction in wake is seen at 3, 10 and 30 mg/kg (Figure 5.13D). Time in NREM sleep was increased following VU0467154 dosing. Like effects on wake, 1 and 3 mg/kg produced transient increases in NREM, with 10 and 30 mg/kg producing more prolonged NREM increases (Figure 5.13B), when assessed across 12 hours, 3, 10 and 30 mg/kg produced

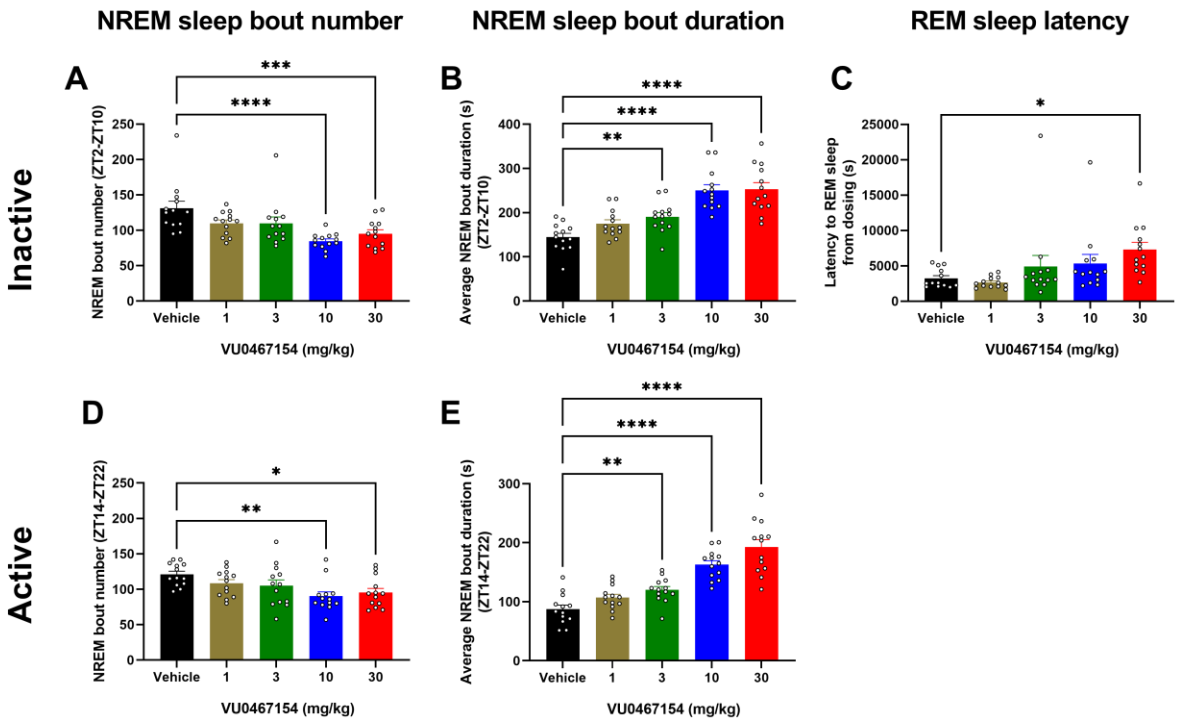

Figure 5.14. The M_4 mAChR PAM VU0467154 reduced sleep fragmentation and during the inactive and active phase increased REM latency during the inactive phase of non-pathologically mice. Shown is the average NREM sleep bout number (A, D), the average NREM sleep bout duration (B, E) and latency to REM sleep (C) in non-pathologically aged mice for 8 hours following dosing in the inactive (A-C) or active (D-E) phase. During the inactive phase VU0467154 dose dependently decreased NREM sleep bout number (A) and increased NREM sleep bout duration (B) and REM sleep latency (C). During the active phase no effect was observed on NREM sleep bout number with a dose dependent increase in NREM sleep bout duration observed. Data are expressed as overall means ± S.E.M., n=13/group. * indicates p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 compared to vehicle (RM 1-way ANOVA followed by Dunnett's test). See table 5.6 for full statistical analysis.

	VU0467154 (mg/kg)						
	Vehicle (SEM)	1 (SEM)	3 (SEM)	10 (SEM)	30 (SEM)	2-Way ANOVA	P value
Inactive (ZT2-ZT10)							
Wake Bout Number	130.4 (10.06)	108.0 (4.655)*	107.4 (9.275)	81.54 (2.690)****	89.92 (5.612)***	$F_{4,48}$ = 8.580	<0.0001
Average Wake Bout Duration (s)	71.78 (5.615)	76.50 (4.839)	72.74 (6.680)	84.27 (9.332)	55.50 (3.360)	$F_{4,48}$ = 2.908	0.0311
Active (ZT14-ZT22)							
Wake Bout Number	125.0 (4.323)	111.4 (18.27)	105.9 (8.152)	90.54 (6.068)***	94.62 (6.051)**	$F_{4,48}$ = 5.087	0.0017
Average Wake Bout Duration (s)	146.6 (10.69)	157.2 (11.63)	168.6 (22.95)	164.5 (17.01)	119.3 (12.17)	$F_{4,48}$ = 1.779	0.1487

Table 5.3. The M_4 mAChR PAM VU0467154 reduced wake bout number in the inactive and active phases in non-pathologically aged mice. Shown is the average wake bout number and average wake duration during the inactive and active phase for the 8-hours following dosing in non-pathologically aged mice. N=13 per group, repeated 2-Way ANOVA comparing dose groups.

increased NREM (Figure 5.13E). VU0467154 reduced REM sleep following dosing. 3 mg/kg reduced REM sleep immediately following dosing with 10 and 30 mg/kg producing more prolonged REM sleep suppression (Figure 5.13C), when assessed across 12 hours, 10 and 30 mg/kg produced decreased REM (Figure 5.13F).

In non-pathologically aged mice dosed in the active period VU0467154 produced a dose dependent reduction in wake. 3 mg/kg produced a transient reduction in wake, with 10 and 30 mg/kg producing a larger reduction in wake (Figure 5.13G), when assessing the effects across 12 hours reduced wake is observed at 10 and 30 mg/kg (Figure 5.13J). VU0467154 produced a dose dependent increase in NREM sleep. 3 mg/kg produced briefly increased NREM sleep and 10 and 30 mg/kg produced larger, sustained increases in NREM sleep (Figure 5.13H), when assessing the effects across 12 hours increased NREM sleep is observed at 10 and 30 mg/kg (Figure 5.13K). Similar to dosing in the young animals, dosing with VU0467154 produced a small but significant increase in REM sleep at 3 and 10 mg/kg, with decreased REM sleep observed 10 hours (10 mg/kg) and 10-14 hours (30 mg/kg) post dosing (Figure 5.13F). When assessing the effects across 12 hours in aged mice, REM sleep is unchanged during the active phase, but decreased at 10 and 30 mg/kg in the inactive phase 12 hours after dosing (Figure 5.13L).

In non-pathologically aged mice, the M_4 mAChR PAM reduced NREM sleep fragmentation across phase and increased latency to REM sleep during the inactive phase.

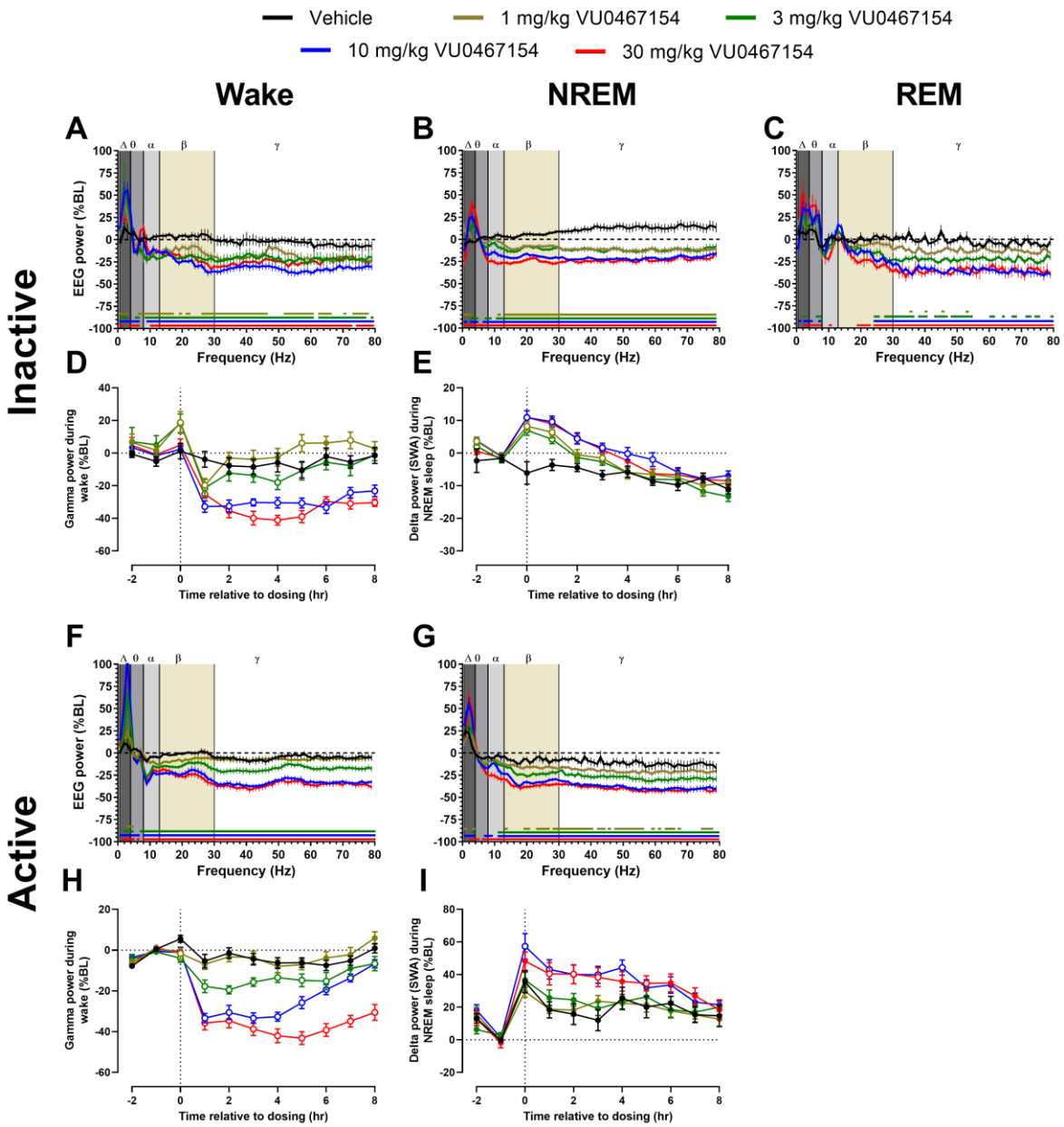

Figure 5.15. The M_4 mAChR PAM VU0467154 produced dose dependent shifts to lower frequencies during all sleep/wake states during the inactive and active phase in non-pathologically aged mice. Shown is the relative spectral power during wake (A, F), NREM sleep (B, G) and REM sleep (C, H) epochs only in the 1-2 hours following compound dosing relative to the 1-hour predose baseline during the inactive phase, and relative gamma power during wake (D, I) and relative delta power (SWA) during NREM sleep (E, J), across the inactive phase in young (A-E) and non-pathologically aged (F-J) mice. In aged mice VU0467154 produced shifts to lower frequencies at all doses tested during wake (A), NREM sleep (B) and REM sleep (C). During wake VU0457154 produced dose-dependent reductions in gamma power (D) and increased relative delta power (SWA) during NREM sleep (E). In aged mice VU0467154 produced shifts to lower frequencies at 3, 10 and 30 mg/kg during wake (F), and at 1, 3, 10 and 30 mg/kg during NREM sleep (G). VU0467154 dose-dependently decreased relative gamma power during wake (H) and 10 and 30 mg/kg VU0467154 increased relative delta power (SWA) during NREM sleep (I). Data are expressed as means ± S.E.M. in 1Hz bins (A-C, F-G) and means in 1hr bins ± S.E.M. (D, E, H, I), n=13/group. Solid bars and open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 5.6 for full statistical analysis.

In non-pathologically aged mice VU0467154 displayed a main effect on NREM bout number and NREM bout duration, with decreased NREM bout number observed at 10 and 30 mg/kg, and increased NREM bout duration observed at 3, 10 and 30 mg/kg (Figure 5.14A, B). This reduction in fragmentation results in dose dependent reductions in wake bout number during the inactive phase in non-pathologically aged mice with significant reductions at 1, 10 and 30 mg/kg. Wake bout duration displays an overall effect of dose in the inactive phase; however, no effect is seen at any individual dose following posthoc comparisons (Table 5.3).

In non-pathologically aged mice during the active phase, VU0467154 reduced NREM sleep bout number with effects at 10 and 30 mg/kg, increased NREM sleep bout length, with increased NREM sleep bout duration observed at 3-30 mg/kg (Fig. 14D, E). Non-pathologically aged mice in contrast to young mice display significantly reduced wake bout number at 10 and

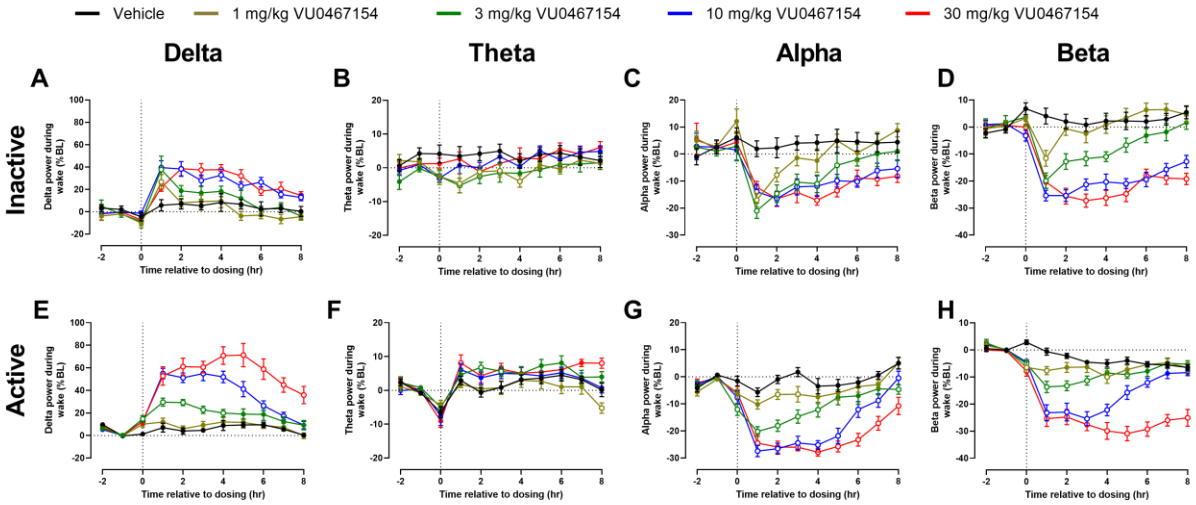

Figure 5.16. The M_4 mAChR PAM VU0467154 produced a dose dependent shift to lower frequency power bands during wake in the inactive and active phase of non-pathologically aged mice. Shown is the change in relative spectral within the delta (A, B), theta (C, D), alpha (E, F) and beta (G, H) powerbands during wake in inactive (A, C, E, G) and active (B, D, F, H) phase of non-pathologically aged mice for 8 hours following VU0467154 dosing 2 hours after light change. VU0467154 dose dependently increased delta power during the inactive (A) and active (E) phase. Theta power was reduced following 1 and 3 mg/kg VU0467154 during the inactive phase (B) and increased following 3 and 30 mg/kg in the active phase (F). VU0467154 dose dependently decreased alpha power during the inactive (C) and active (G) phase. VU0467154 dose dependently decreased beta power in the inactive (D) and active (H) phase. Data are expressed as means in 1hr bins ± S.E.M., n=13/group. Open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 5.6 for full statistical analysis.

30 mg/kg with no effect of dose on wake bout duration seen (Table 5.3). In non-pathologically aged mice, VU0467154 increased REM sleep latency following dosing, with significance observed at 30 mg/kg (Fig. 14C).

In non-pathologically aged mice, the M_4 mAChR PAM VU0467154 produced a shift to lower frequencies during all sleep states during both the inactive and active phases

During the inactive phase in non-pathologically aged mice during wake, VU0467154 produced a shift to lower frequencies, with increased delta power and reduced theta, alpha, beta and gamma powers were seen at all doses (Figure 5.15A), resulting in dose dependently decreased gamma power (Figure 5.15D). Consistent with this shift to lower frequencies reduced theta, alpha and beta frequencies and increased delta frequencies are seen across the inactive

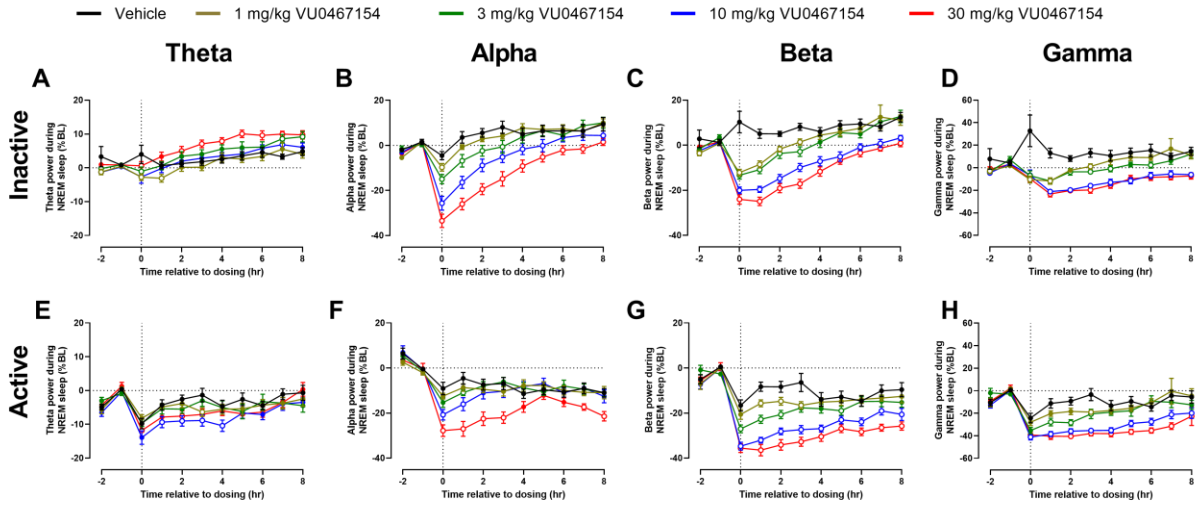

Figure 5.17. The M_4 mAChR PAM VU0467154 produced a dose dependent shift to lower frequency power bands during NREM sleep in the inactive and active phase of non-pathologically aged mice. Shown is the change in relative spectral within the theta (A, B), alpha (C, D), beta (E, F) and gamma (G, H) powerbands during NREM sleep in inactive (A, C, E, G) and active (B, D, F, H) phase of non-pathologically aged mice for 8 hours following VU0467154 dosing 2 hours after light change. VU0467154 increased theta power following 30 mg/kg VU0467154 during the inactive phase (A) and reduced theta power following 10 and 30 mg/kg VU0467154 during the active phase (E). VU0467154 dose dependently decreased alpha power during the inactive (B) and the active (F) phase. VU0467154 dose dependently decreased beta power during the inactive (C) and active (G) phase. VU0467154 dose dependently decreased gamma power in the inactive (D) and active (H) phase. Data are expressed as means in 1hr bins ± S.E.M., n=13/group. Open symbols indicate $p<0.05$ compared to vehicle (RM 2-way ANOVA matching by both factors followed by Dunnett's test). See table 5.6 for full statistical analysis.

phase (Figure 5.16). VU0467154 dosed in the inactive phase produced a shift to lower frequencies during NREM sleep in aged mice. 1 mg/kg reduced lower delta frequencies, with 3-30 mg/kg increasing higher delta frequencies, and 10 and 30 mg/kg reducing lower delta frequencies. All doses reduced beta and gamma powers, with 10 mg/kg also reducing alpha power and 30 mg/kg reducing theta and alpha powers (Figure 5.15B). All doses increased delta power during NREM sleep in aged mice (Figure 5.15E). Consistent with this shift to lower frequencies increased theta, and reduced alpha, beta and gamma frequencies are observed (Figure 5.17). During REM sleep in aged mice 3, 10 and 30 mg/kg VU0467154 increased delta and theta frequencies, while 3, 10 and 30 mg/kg decreased alpha and gamma frequencies.

In non-pathologically aged animals dosed with VU0467154 in the active period a shift to lower frequencies during wake epochs is observed, all doses increased delta power and 3, 10 and 30 mg/kg decreased alpha, beta and gamma powers (Figure 5.15F), resulting in a dose-dependent decrease in gamma frequency during wake across the active phase (Figure 5.15H). Consistent with this shift to lower frequencies during wake epochs, VU0467154 produced increased delta and theta power and reduced alpha and beta power across the inactive phase (Figure 5.16). In non-pathologically aged mice dosed in the active period a shift to lower frequencies is also seen during NREM sleep epochs, all doses increased delta power, with a dose dependent reduction in beta and gamma powers, and reduced alpha power observed (Figure 5.15G). VU0467154 dose dependently increased delta power (SWA) during NREM sleep with significance observed at 10 and 30 mg/kg (Figure 5.15I). Consistent with this shift to lower frequencies during NREM sleep epochs during the active phase, VU0467154 produced decreased theta, alpha, beta and gamma power across the inactive phase (Figure 5.17).

The M_4 mAChR PAM VU0467154 dosed in the inactive and active phases produced no cholinergic side effects in aged mice and pharmacokinetic analysis revealed comparable free brain VU04567154 concentrations in young and aged mice.

	Young[a]	Aged
T_{max}	1	Not defined
[Plasma] 1hr post dose (S.E.M.) (μM)	2.0	7.72 (1.34)
Brain/Plasma K_p (S.E.M.)	0.64[b]	0.21[c] (0.016)
Brain/Plasma $K_{p,uu}$	0.41[b]	0.13[c]
Free Brain 1hr post-dose (nM)	17.9	21.8

Table 5.4. VU0467154 pK following 3mg/kg dose IP in aged mice

When aged mice were dosed in the inactive or active periods at the highest dose of 30mg/kg, VU0467154 dosed mice displayed no difference to vehicle dosed mice, with no cholinergic side effects seen (Table 5.5). Pharmacokinetic assessment in aged animals revealed a higher plasma concentration at 1 hour post dosing than previously seen in young mice (Bubser et al., 2014), however the plasma-brain ratio was reduced a similar fold resulting in comparable free brain concentrations in young and old mice (Table 5.4).

5.4. Discussion

The muscarinic cholinergic system is known to be important in modulating sleep-wake architecture with M_1 having a role in promoting wake and arousal (Gould et al., 2020), M_1, M_2 and M_3 being shown to be important in REM sleep modulation (Coleman et al., 2004; Gould et al., 2020; Niwa et al., 2018). However, knockout data indicate that the M_4 mAChR does not play a major role in modulating sleep-wake architecture (Goutagny et al., 2005; Turner et al., 2010). More recent work with M_4 mAChR selective ligands has suggested that M_4 stimulation reduces REM sleep and increases total sleep time (Gould et al., 2016). The current data provide evidence that selective M_4 antagonist VU6028418 attenuates the effects of the M_4 mAChR PAM VU0467154 on sleep/wake architecture and arousal in young mice. This suggests that M_4 mAChR activation plays a role in increasing NREM sleep, reducing REM sleep, and reducing arousal. With M_4 mAChR activation being explored as an exciting treatment in schizophrenia and behavioral disturbances in AD it is important to understand how these effects may differ in young and aged preclinical species and clinical populations.

Following combination dosing with the M_4 mAChR PAM VU0467154 and the M_4 mAChR antagonist VU6028418, VU0467154 dependent increases in NREM sleep duration and fragmentation, and reductions in gamma power during wake are robustly reversed. Interestingly, the VU0467154 dependent REM sleep reduction is attenuated with no effect observed on the VU0467154 dependent increase in REM sleep latency. We hypothesize that this is due to the differing pharmacological mechanisms of the two compounds. While VU0467154 binds allosterically, VU6028418 binds orthosterically and due to the lack of a competitive interaction both compounds could bind simultaneously to the receptor and produce conflicting effects. VU0467154 NREM sleep promoting effects and VU6028418 promoting wake resulting in the mice not entering a sufficiently deep NREM sleep to progress to REM sleep. This is supported by the profound decrease in delta power (SWA) during NREM sleep following combination dosing with both compounds in the inactive phase. Despite this conflicting action at the receptor, we observe an attenuation of the VU0467154 dependent reduction in REM sleep suggesting the reduction in REM sleep is M_4-dependent.

Having confirmed the effects of the M_4 mAChR PAM VU0467154 were M_4 dependent, it is important to test if these effects would translate to non-pathologically aged mice where cholinergic signaling is altered (Mitsushima et al., 1996), as this would have important implications for the efficacy of on M_4 mAChR PAM as individuals ages or in a disease associated with advanced age. Similar to our previous data, we demonstrated that non-pathologically aged mice display reduced REM sleep (Gould et al., 2020), wake fragmentation during the active phase, and reduced arousal (Russell et al., 2023). In the current data set decreased arousal was observed across phase compared to just in the active phase in our previous work, this is presumably due to the mice being older in these studies than previously. In the present study all the M_4 dependent effects observed in young mice were also observed in non-pathologically aged mice. Additionally, we observed that the M_4 mAChR PAM produced more robust increases in delta power (SWA)

during NREM sleep across phase in non-pathologically aged mice than was observed in young mice.

Sleep/wake architecture is known to be disrupted in many neuropsychiatric conditions. In schizophrenia, patient populations display decreased REM latency, decreased NREM sleep duration, decreased NREM delta power (SWA), and increased sleep fragmentation (Chan et al., 2017; Das et al., 2005; Kasanova et al., 2020; Kaskie et al., 2019). Sleep disturbances have been suggested to exacerbate symptoms such as paranoia (Ferrarelli, 2021; Kasanova et al., 2020), and it has been suggested that normalizing observed sleep/wake architecture abnormalities in schizophrenic patients may produce improvements in other symptom clusters (Manoach and Stickgold, 2009) The M_4 mAChR has been investigated as an exciting target for the treatment of schizophrenia (Foster et al., 2014; C. K. Jones et al., 2012). In the present study we demonstrated selective activation of the M_4 mAChR during the active and inactive phase increased time spent in NREM sleep, reducing NREM sleep fragmentation and decreased time spent in wake. These findings suggest that the M_4 mAChR mediated effects on NREM sleep may be beneficial in normalizing NREM sleep disturbances in schizophrenia. During the inactive phase the M_4 mAChR PAM VU0467154 dose dependently decreased REM sleep in young and aged mice, with increased REM latency in young animals. In schizophrenia REM latency is reduced and has been correlated with the severity of negative symptoms and neurocognitive symptoms (Chan et al., 2017; Das et al., 2005), suggesting that increasing REM latency in patients with schizophrenia may modulate negative and cognitive symptom clusters.

In addition to treatment of schizophrenia, M_4 activation is being pursued as a potential treatment for the behavioral disturbances in AD (Foster et al., 2014) (ClinicalTrials.gov: NCT05511363). In AD patients reduced NREM sleep quality and quantity is observed (Bubu et al., 2017; Prinz, Vitaliano, et al., 1982), with increased NREM sleep fragmentation (Peter-Derex et al., 2015). Furthermore, decreased NREM sleep has been linked to increased pathology in AD (Bubu et al., 2017; Shokri-Kojori et al., 2018; Yulug et al., 2017). It has been suggested that

normalizing sleep deficits may provide a novel approach for disease modification in AD (Y. F. Lee et al., 2020; C. Wang and Holtzman, 2020), as studies have demonstrated that the glymphatic system is responsible for the clearance of soluble β-amyloid and tau oligomers and is most effective during NREM sleep and (Iliff et al., 2012, 2014). Future studies will investigate whether M_4 mAChR dependent NREM sleep enhancement may provide an avenue to enhancing glymphatic activity. Patients with AD display reduced REM sleep (Y. Zhang et al., 2022), as such further reductions as observed in the present study may not be desirable. However, individuals who are treated with AChEIs display increased REM sleep (Moraes et al., 2006), which in some individuals has been associated with increased nightmares (Dunn et al., 2000; Ridha et al., 2018), suggesting M_4 mediated reductions in REM sleep may have benefit as an adjunct therapy with AChEIs in a subset of AD patients.

In schizophrenia it has been demonstrated that increased gamma power is associated with positive symptoms (Baldeweg et al., 1998; Yadav et al., 2021), with shifts to lower powers, with reduced gamma power associated with poorer cognitive performance (C. M. A. Chen et al., 2014). In AD, patients exhibit a shift to slower powers, which is associated with the transition to dementia, AD pathology and poorer cognitive performance (Cecchetti et al., 2021; Claus et al., 1998; Hamilton et al., 2021). In all arousal states (wake, NREM and REM), during the active and inactive phases, the M_4 mAChR PAM VU0467154 produced shifts from higher frequencies to lower frequencies, with reduced gamma power. The observed shift to lower powers during wake observed with VU0467154 may be beneficial in the treatment of the positive symptoms associated with schizophrenia, but detrimental in the treatment of the cognitive symptoms in schizophrenia and AD. However, we have previously demonstrated VU0467154 enhances cognition in mice (Bubser et al., 2014; Gould et al., 2018), and increases gamma power, a correlate of arousal, in rats during wake (Gould et al., 2016). These differences in gamma power modulation may be due to methodological differences, in our previous work in rats a frontal cortical lead was used for the assessment of acute drug challenges, while in the present study a frontal-parietal lead

configuration was used. Alternatively, these differences may be species dependent in which case further studies in higher order species will be required to establish which best translates to clinical populations.

These data suggest that positive M_4 modulation remains an exciting target for the treatment of schizophrenia in both young and older age, with M_4-dependent effects on NREM sleep, REM sleep and gamma power which may all have therapeutic benefit in patients with schizophrenia. Treatment of the behavioral disturbances in AD with an M_4 mAChR PAM displays a more complex relationship with the wider AD symptomatology. While desirable effects are seen in non-pathologically aged mice with increased NREM sleep and delta power (SWA) during NREM sleep, effects during wake and on REM sleep may be less desirable. AChEIs disrupt NREM sleep and promote REM sleep and so M_4 mAChR PAMs may be beneficial in a subset of AD patients in combination with AChEIs.

	INACTIVE								ACTIVE							
	Vehicle				30 mg/kg VU0467154				Vehicle				30 mg/kg VU0467154			
Time (minutes)	30	60	120	240	30	60	120	240	30	60	120	240	30	60	120	240
Autonomic Nervous System																
Ptosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exophtalmus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corneal reflex loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pinna reflex loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Respiratory rate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Writing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tail erection	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lacrimation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vasodilation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Skin color	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Irritability	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Baseline pupil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pupil reaction	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Somatomotor Systems																
Motor activity	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arch/Roll	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leg weakness	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rigid stance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spraddle	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Placing loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Grasping loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Righting loss	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Catalepsy	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tail pinch	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	**0.000**	**0.400**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	Dose $F_{(1, 8)}$=1.000, p=0.4079; Time $F_{(3, 27)}$=1.000, p=0.3466								Dose $F_{(1, 8)}$=0.000, p>0.9999; Time $F_{(3, 27)}$=0.000, p>0.9999 (groups identical)							
	For all behaviors scored: 0 = normal, 1 = mild effect and 2 = severe effect															

Table 5.5. VU0467154 does not produce cholinergic adverse effects in non-pathologically aged mice.

Figure	Age	Experiment	Measure	Phase	Statistical Test	Comparison	Degrees of freedom	F or T	P	*	Group Size	Post hoc results
1a	Young	VU0467154 effects on time in w ake	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	23.48	<0.0001	****	14	10 mg/kg vs Veh time: ZT4 30 mg/kg vs Veh time: ZT4, 6, 10, 12, 16 and 18
						Time	11, 143	154.0	<0.0001	****		
						Dose x Time	44, 572	2.647	<0.0001	****		
1b	Young	VU0467154 effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	28.88	<0.0001	****	14	1mg/kg vs Veh time: ZT4 10 mg/kg vs Veh time: ZT4 30 mg/kg vs Veh time: ZT4, 6, 10, 12, 16 and 18
						Time	11, 143	156.7	<0.0001	****		
						Dose x Time	44, 572	3.278	<0.0001	****		
1c	Young	VU0467154 effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	8.176	<0.0001	****	14	1mg/kg vs Veh time: ZT2 and 6 10 mg/kg vs Veh time: ZT2, 4 and 6 30 mg/kg vs Veh time: ZT 2, 4, 6, 8, 16 and 18
						Time	11, 143	79.60	<0.0001	****		
						Dose x Time	44, 572	5.311	<0.0001	****		
1d	Young	VU0467154 effects on time in w ake	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	23.48	<0.0001	****	14	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12, ZT12-24
						Time	1, 13	873.1	<0.0001	****		
						Dose x Time	4, 52	3.597	0.0116	*		
1e	Young	VU0467154 effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	28.88	<0.0001	****	14	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12, ZT12-24
						Time	1, 13	915.4	<0.0001	****		
						Dose x Time	4, 52	4.714	0.0025	**		
1f	Young	VU0467154 effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	8.176	<0.0001	****	14	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12, ZT12-24
						Time	1, 13	327	<0.0001	****		
						Dose x Time	4, 52	14.79	<0.0001	****		
1g	Young	VU0467154 / VU6028418 effects on time in w ake	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	87.83	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT2, 4, 6, 12 and 14 Veh/Veh vs Veh/VU418 10 mg/kg: ZT2 and 18 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 2 and 4 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 2, 4, 6, 12, 14 and 16
						Time	11, 143	103.4	<0.0001	****		
						Dose x Time	33, 429	7.965	<0.0001	****		
1h	Young	VU0467154 / VU6028418 effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	100.3	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT2, 4, 6, 8, 10, 12, 14 and 20 Veh/Veh vs Veh/VU418 10 mg/kg: ZT2, 16 and 18 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 2 and 4 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 2, 4, 6, 8, 10, 12, 14, 16 and 18
						Time	11, 143	111.6	<0.0001	****		
						Dose x Time	33, 429	8.566	<0.0001	****		
1i	Young	VU0467154 / VU6028418 effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	14.00	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT4, 6, 8 and 10 Veh/Veh vs Veh/VU418 10 mg/kg: ZT18 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 4, 6, 14 and 20 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 6, 8, 10 and 20
						Time	11, 143	43.94	<0.0001	****		
						Dose x Time	33, 429	14.34	<0.0001	****		
1j	Young	VU0467154 / VU6028418 effects on time in w ake	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	87.83	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT0-12 and 12-24 Veh/Veh vs Veh/VU418 10 mg/kg: ZT12-24 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12 and 12-24
						Time	1, 13	321.1	<0.0001	****		
						Dose x Time	3, 39	13.24	<0.0001	****		
1k	Young	VU0467154 / VU6028418 effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	100.3	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT0-12 and 12-24 Veh/Veh vs Veh/VU418 10 mg/kg: ZT12-24 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12 and 12-24
						Time	1, 13	327.1	<0.0001	****		
						Dose x Time	3, 39	12.91	<0.0001	****		
1l	Young	VU0467154 / VU6028418 effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	14.00	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT0-12 Veh/Veh vs Veh/VU418 10 mg/kg: ZT12-24 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12 and 12-24 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12
						Time	1, 13	42.68	<0.0001	****		
						Dose x Time	3, 39	56.68	<0.0001	****		
2a	Young	VU0467154 effects on NREM Bout #	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	3.848	0.0082	**	14	10 mg/kg vs Veh NREM bout number: P=0.0041 30 mg/kg vs Veh NREM bout number: P=0.0088
2b	Young	VU0467154 effects on NREM Bout duration	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	8.698	<0.0001	****	14	10 mg/kg vs Veh NREM bout duration: P=0.0012 30 mg/kg vs Veh NREM bout duration: P=<0.0001
2c	Young	VU0467154 effects on REM sleep latency	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	19.46	<0.0001	****	14	10 mg/kg vs Veh REM latency: P=0.0183 30 mg/kg vs Veh REM latency: P<0.0001
2d	Young	VU0467154 / VU6028418 effects on NREM Bout #	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	23.21	<0.0001	****	14	Veh/Veh vs Veh/VU418 10 mg/kg: p=0.0162 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p<0.0001 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p<0.0001
2e	Young	VU0467154 / VU6028418 effects on NREM Bout duration	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	41.26	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: p<0.0001 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p=0.0260 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p<0.0001
2f	Young	VU0467154 / VU6028418 effects on REM latency	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	3, 39	20.59	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: p<0.0001 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p<0.0001

3a	Young	VU0467154 effects on time in w ake	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	74.03	<0.0001	****	14	1mg/kg vs Veh time: ZT14 and 16 3 mg/kg vs Veh time: ZT14 and 16 10 mg/kg vs Veh time: ZT14, 16 18 and 20 30 mg/kg vs Veh time: ZT4, 14, 16 18, 20 and 22
						Time	11, 143	93.96	<0.0001	****		
						Dose x Time	44, 572	7.357	<0.0001	****		
3b	Young	VU0467154 effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	81.44	<0.0001	****	14	1mg/kg vs Veh time: ZT14 and 16 3 mg/kg vs Veh time: ZT14 and 16 10 mg/kg vs Veh time: ZT14,16, 18 and 20 30 mg/kg vs Veh time: ZT14,16, 18, 20 and 22
						Time	11, 143	89.54	<0.0001	****		
						Dose x Time	44, 572	7.839	<0.0001	****		
3c	Young	VU0467154 effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	6.441	<0.0001	****	14	1mg/kg vs Veh time: ZT16 3 mg/kg vs Veh time: ZT0 and 4 10 mg/kg vs Veh time: ZT0 and 4 30 mg/kg vs Veh time: ZT0, 2, 4, 8 and 20
						Time	11, 143	94.79	0.0003	***		
						Dose x Time	44, 572	3.442	<0.0001	****		
3d	Young	VU0467154 effects on time in w ake	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	74.03	<0.0001	****	14	1mg/kg vs Veh time: ZT12-24 3 mg/kg vs Veh time: ZT12-24 10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24, ZT0-12
						Time	1, 13	346.8	<0.0001	****		
						Dose x Time	4, 52	80.49	<0.0001	****		
3e	Young	VU0467154 effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	81.44	<0.0001	****	14	1mg/kg vs Veh time: ZT12-24 3 mg/kg vs Veh time: ZT12-24 10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24
						Time	1, 13	315.55	<0.0001	****		
						Dose x Time	4, 52	85.62	<0.0001	****		
3f	Young	VU0467154 effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	4, 52	6.441	0.0003	***	14	10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24, ZT0-12
						Time	1, 13	395.7	<0.0001	****		
						Dose x Time	4, 52	12.88	<0.0001	****		
3g	Young	VU0467154 / VU6028418 effects on time in w ake	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	124.3	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT14, 16, 18, 20 and 22 Veh/Veh vs Veh/VU418 10 mg/kg: ZT14, 18, 22 and 0 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 14, 16, 18 and 22 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 14, 16, 18, 20 and 22
						Time	11, 143	123.1	<0.0001	****		
						Dose x Time	33, 429	12.07	<0.0001	****		
3h	Young	VU0467154 / VU6028418 effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	114.0	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT14, 16, 18, 20 and 22 Veh/Veh vs Veh/VU418 10 mg/kg: ZT14, 22, 0 and 8 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 14, 16, 18 and 22 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 14, 16, 18, 20 and 22
						Time	11, 143	108.2	<0.0001	****		
						Dose x Time	33, 429	12.56	<0.0001	****		
3i	Young	VU0467154 / VU6028418 effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	2.690	0.0595	ns	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT18, 0 and 2 Veh/Veh vs Veh/VU418 10 mg/kg: ZT18, 0, 4 and 10 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 18 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT 20 and 2
						Time	11, 143	217.6	<0.0001	****		
						Dose x Time	33, 429	5.412	<0.0001	****		
3j	Young	VU0467154 / VU6028418 effects on time in w ake	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	124.3	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT0-12 Veh/Veh vs Veh/VU418 10 mg/kg: ZT0-12, 12-24 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12
						Time	1, 13	965.1	<0.0001	****		
						Dose x Time	3, 39	51.03	<0.0001	****		
3k	Young	VU0467154 / VU6028418 effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	114.0	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT0-12 Veh/Veh vs Veh/VU418 10 mg/kg: ZT0-12, 12-24 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12
						Time	1, 13	905.3	<0.0001	****		
						Dose x Time	3, 39	44.75	<0.0001	****		
3l	Young	VU0467154 / VU6028418 effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Tw o-Way ANOVA	Dose	3, 39	2.690	0.0595	ns	14	Veh/Veh vs VU154 30mg/kg/Veh: ZT12-24 Veh/Veh vs Veh/VU418 10 mg/kg: ZT0-12, 12-24 Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT12-24 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: ZT0-12, 12-24
						Time	1, 13	1457	<0.0001	****		
						Dose x Time	3, 39	32.52	<0.0001	****		
4a	Young	VU0467154 effects on NREM Bout #	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	4.010	0.0066	**	14	1mg/kg vs Veh NREM bout number: P=0.0385
4b	Young	VU0467154 effects on NREM Bout duration	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	4.881	<0.0001	****	14	10 mg/kg vs Veh NREM bout duration: P=0.0002 30 mg/kg vs Veh NREM bout duration: P<0.0001
4c	Young	VU0467154 / VU6028418 effects on NREM Bout #	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	5.260	0.0038	**	14	VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p=0.0094
4d	Young	VU0467154 / VU6028418 effects on NREM Bout duration	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	3, 39	71.56	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: p<0.0001 Veh/Veh vs Veh/VU418 10 mg/kg: p=0.0039 VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: p<0.0001

5a	Young	VU0467154 effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	9.958	<0.0001	****	14	1mg/kg vs Veh Freq: 2-4, 25, 28, 30-31, 33-36 40-54, 58, 60-69, 71and 73-79Hz 3 mg/kg vs Veh Freq: 2-4, 6, 9-21, 25, 31, 42, 50-51Hz 10 mg/kg vs Veh Freq: 2-4, 6, 9-57, 60-61, 65, and 67-79Hz 30 mg/kg vs Veh Freq: 2-4, 6, 9-79Hz
						Frequency	79, 1027	35.49	<0.0001	****		
						Dose x Frequency	316, 4108	4.859	<0.0001	****		
5b	Young	VU0467154 effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	5.656	0.0007	***	14	1mg/kg vs Veh Freq: 0.5, 3, 25-28, 35-37, 44-46, 48 and 57Hz 3 mg/kg vs Veh Freq: 2-3, 7-9, 12-62, 64 and 79Hz 10 mg/kg vs Veh Freq: 0.5-5, 7-9, 11-28, 30-78 Hz 30 mg/kg vs Veh Freq: 0.5-5, 7-32, 35-54, 57-62, 65, 72 and 77Hz
						Frequency	79, 1027	14.53	<0.0001	****		
						Dose x Frequency	316, 4108	7.174	<0.0001	****		
5c	Young	VU0467154 effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	27.02	<0.0001	****	7-14	1mg/kg vs Veh Freq: 2, 8, 28 and 75Hz 3 mg/kg vs Veh Freq: 2-6, 8, 19-21, 23-25, 27-79Hz 10 mg/kg vs Veh Freq: 2-6, 8, 18-79Hz 30 mg/kg vs Veh Freq: 2-5, 7, 13, 19-20, 23-79Hz
						Frequency	79, 1027	45.55	<0.0001	****		
						Dose x Frequency	316, 3388	6.325	<0.0001	****		
5d	Young	VU0467154 effects on gamma power during wake	% change from BL	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	7.848	<0.0001	****	14	1mg/kg vs Veh time: 0, 1, 2, 3, 5 and 7hrs 3 mg/kg vs Veh time: 2 and 7hrs 10 mg/kg vs Veh time: 1, 2, 3, 4, 5 and 7hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	31.89	<0.0001	****		
						Dose x Time	40, 520	5.062	<0.0001	****		
5e	Young	VU0467154 effects on NREM delta (SWA)	% change from BL	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	3.084	0.0236	*	14	1mg/kg vs Veh time: 0hrs 3 mg/kg vs Veh time: 0, 1, 3, 4, 5 and 8hrs 10 mg/kg vs Veh time: 0hr 30 mg/kg vs Veh time: 1hr
						Time	10, 130	121.3	<0.0001	****		
						Dose x Time	40, 520	2.437	<0.0001	****		
5f	Young	VU0467154 / VU6028418 effects on wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	19.20	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 4, 6, 11, 19-23, 27-34, 42, 44-45, 47, 56 and 79Hz Veh/Veh vs Veh/VU418 10 mg/kg: 3-4, 38-46, 48-50, 53, 59-62 and 67Hz Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 2-3, 11, 39, 41, 47, 54 and 57-79Hz VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0.5-2, 4, 6, 10-23 and 63Hz
						Frequency	79, 1027	14.96	<0.0001	****		
						Dose x Frequency	237, 3081	11.73	<0.0001	****		
5g	Young	VU0467154 / VU6028418 effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	64.52	<0.0001	****	12-14	Veh/Veh vs VU154 30mg/kg/Veh: 0.5-1, 3-4, 9-20, 24-30, 35-41, 47-51, 58-60 and 70-71Hz Veh/Veh vs Veh/VU418 10 mg/kg: 32, 37-38 and 43-79Hz Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0.5-1and 20-79Hz VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 3-4, 8-11and 15-79Hz
						Frequency	79, 1027	32.27	<0.0001	****		
						Dose x Frequency	237, 2921	18.71	<0.0001	****		
5h	Young	VU0467154 / VU6028418 effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	1, 13	0.08982	0.7691	ns	9-13	Veh/Veh vs Veh/VU418 10 mg/kg: 3Hz
						Frequency	79, 1027	3.369	<0.0001	****		
						Dose x Frequency	79, 547	2.316	<0.0001	****		
5i	Young	VU0467154 / VU6028418 effects on gamma power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	28.17	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: -1, 0, 1, 2, 3, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1and 2hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	16.81	<0.0001	****		
						Dose x Time	30, 390	9.139	<0.0001	****		
5j	Young	VU0467154 / VU6028418 effects on NREM delta (SWA)	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	2.090	0.1173	ns	14	Veh/Veh vs VU154 30mg/kg/Veh: 0hrs Veh/Veh vs Veh/VU418 10 mg/kg:0hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1and 2hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1and 2hrs
						Time	10, 130	30.84	<0.0001	****		
						Dose x Time	30, 375	7.767	<0.0001	****		
6a	Young	VU0467154 effects on delta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	9.118	<0.0001	****	14	1mg/kg vs Veh time: 0, 1, 2, 3, 4 and 7hrs 3 mg/kg vs Veh time: 1, 2 and 7hrs 10 mg/kg vs Veh time: 1, 2, 3, 4 and 5hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	42.96	<0.0001	****		
						Dose x Time	40, 520	4.582	<0.0001	****		
6b	Young	VU0467154 effects on theta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	4.600	0.0030	**	14	1mg/kg vs Veh time: 0, 1, 2, 3, 5, 6 and 7hrs 3 mg/kg vs Veh time: 2, 3, 6 and 7hrs 10 mg/kg vs Veh time: 3 and 7hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	24.46	<0.0001	****		
						Dose x Time	40, 520	3.155	<0.0001	****		
6c	Young	VU0467154 effects on alpha power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	3.931	0.0073	**	14	1mg/kg vs Veh time: 0, 1, 4 and 7hrs 3 mg/kg vs Veh time: 1, 2 and 3hrs 10 mg/kg vs Veh time: 0, 1, 2, 3 and 4hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	35.65	<0.0001	****		
						Dose x Time	40, 520	3.727	<0.0001	****		
6d	Young	VU0467154 effects on beta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	18.78	<0.0001	****	14	1mg/kg vs Veh time: -2, 0, 1and 2hrs 3 mg/kg vs Veh time: 1, 2, 3, 4, 5 and 7hrs 10 mg/kg vs Veh time:0, 1, 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	27.79	<0.0001	****		
						Dose x Time	40, 520	10.64	<0.0001	****		
6e	Young	VU0467154 / VU6028418 effects on wake delta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	10.14	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: -1, 0, 1and 2hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: -1, 0, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	9.163	<0.0001	****		
						Dose x Time	30, 390	6.139	<0.0001	****		
6f	Young	VU0467154 / VU6028418 effects on wake theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	5.675	0.0025	**	14	Veh/Veh vs VU154 30mg/kg/Veh: 5 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 4, 5, 6, 7 and 8hrs
						Time	10, 130	6.177	<0.0001	****		
						Dose x Time	30, 390	1.796	0.0072	**		
6g	Young	VU0467154 / VU6028418 effects on wake alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	4.912	0.0054	**	14	Veh/Veh vs VU154 30mg/kg/Veh: 1, 2, 3, 4 and 5hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: -1, 1and 3hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: -1, 1, 2, 3, 4 and 5hrs
						Time	10, 130	5.956	<0.0001	****		
						Dose x Time	30, 390	3.915	<0.0001	****		
6h	Young	VU0467154 / VU6028418 effects on NREM beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	49.30	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0 and 8hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	14.89	<0.0001	****		
						Dose x Time	30, 390	121.28	<0.0001	****		

7a	Young	VU0467154 effects on NREM theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	5.691	0.0007	***	14	1mg/kg vs Veh time: 0hrs 10 mg/kg vs Veh time: 1, 2, 3, 4, 5, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	53.44	<0.0001	****		
						Dose x Time	40, 520	2.641	<0.0001	****		
7b	Young	VU0467154 effects on NREM alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	7.644	<0.0001	****	14	1mg/kg vs Veh time: 0, 1and 6hrs 3 mg/kg vs Veh time: 0, 1and 8hrs 10 mg/kg vs Veh time: 0 and 1hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5 and 6hrs
						Time	10, 130	117.0	<0.0001	****		
						Dose x Time	40, 520	10.43	<0.0001	****		
7c	Young	VU0467154 effects on NREM beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	5.175	0.0014	**	14	1mg/kg vs Veh time: 1hrs 3 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5 and 8hrs 10 mg/kg vs Veh time: -1.0, 1, 2 and 3hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	84.21	<0.0001	****		
						Dose x Time	40, 520	5.324	<0.0001	****		
7d	Young	VU0467154 effects on NREM gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 52	4.291	0.0045	**	14	1mg/kg vs Veh time: 0 and 4hrs 3 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6 and 7hrs 10 mg/kg vs Veh time: -1, 1, 2, 3, 4, 5 and 6hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	32.99	<0.0001	****		
						Dose x Time	40, 520	7.685	<0.0001	****		
7e	Young	VU0467154 / VU6028418 effects on NREM theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	17.75	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1, 2 and 3hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1, 4, 5, 6, 7 and 8hrs
						Time	10, 130	15.41	<0.0001	****		
						Dose x Time	30, 375	5.245	<0.0001	****		
7f	Young	VU0467154 / VU6028418 effects on NREM alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	12.24	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: 0hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 2, 3 and 4hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1, 2 and 5hrs
						Time	10, 130	65.45	<0.0001	****		
						Dose x Time	30, 375	6.856	<0.0001	****		
7g	Young	VU0467154 / VU6028418 effects on NREM beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	29.93	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: -1.0 and 1hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0 and 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1, 2, 3, 4, 5 and 8hrs
						Time	10, 130	26.44	<0.0001	****		
						Dose x Time	30, 375	11.98	<0.0001	****		
7h	Young	VU0467154 / VU6028418 effects on NREM gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	3, 39	32.04	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 2 and 7hrs Veh/Veh vs Veh/VU418 10 mg/kg: 0 and 1hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1, 2 and 3hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1, 2, 3, 4, 7 and 8hrs
						Time	10, 130	30.72	<0.0001	****		
						Dose x Time	30, 375	13.55	<0.0001	****		
8a	Young	VU0467154 effects on NREM qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	21.16	<0.0001	****	14	1mg/kg vs Veh Freq: 2-4, 8-10, 24-25, 40-49 and 60-79Hz 3 mg/kg vs Veh Freq: 2-5, 7-11, 26, 28-79Hz 10 mg/kg vs Veh Freq: 0.5-5, 7-13, 18-21and 26-79Hz 30 mg/kg vs Veh Freq: 2-4, 6-7, 9-22, 28-79Hz
						Frequency	79, 1027	41.57	<0.0001	****		
						Dose x Frequency	316, 4108	9.005	<0.0001	****		
8b	Young	VU0467154 effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	6.253	0.0003	***	13-14	1mg/kg vs Veh Freq: 61, 64Hz 3 mg/kg vs Veh Freq: 0.5-2, 5-6Hz 10 mg/kg vs Veh Freq: 0.5-3Hz 30 mg/kg vs Veh Freq: 0.5-1.3-5, 8-33, 35-41.45-49, 52, 54-66 and 69-75Hz
						Frequency	79, 1027	93.45	<0.0001	****		
						Dose x Frequency	316, 4028	4.845	<0.0001	****		
8c	Young	VU0467154 effects on gamma power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	29.72	<0.0001	****	14	1mg/kg vs Veh time: 1hrs 3 mg/kg vs Veh time: 1, 2, 3 and 5hrs 10 mg/kg vs Veh time: 1, 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	37.19	<0.0001	****		
						Dose x Time	40, 520	7.317	<0.0001	****		
8d	Young	VU0467154 effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	1.967	0.1134	ns	14	1mg/kg vs Veh time: 4, 5 and 6hrs 3 mg/kg vs Veh time: 1and 7hrs 10 mg/kg vs Veh time: 4, 7 and 8hrs
						Time	10, 130	47.09	<0.0001	****		
						Dose x Time	40, 520	2.876	<0.0001	****		
8e	Young	VU0467154 / VU6028418 effects on wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	16.55	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 2-5, 9-15, 17-79Hz Veh/Veh vs Veh/VU418 10 mg/kg: 2-3, 8, 71-73 and 75-79Hz Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 2-4, 10, 39-49, 62-79Hz VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 3-4, 10-11, 20-23, 26-60, 62 and 75-79Hz
						Frequency	79, 1027	14.67	<0.0001	****		
						Dose x Frequency	237, 3081	5.593	<0.0001	****		
8f	Young	VU0467154 / VU6028418 effects on NREM qEEG	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	7.058	0.0007	***	12-14	Veh/Veh vs Veh/VU418 10 mg/kg: 0.5, 35, 40, 42, 44-46, 51-66, 68-76 and 78-79Hz VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1Hz
						Frequency	79, 1027	6.649	<0.0001	****		
						Dose x Frequency	237, 2681	3.502	<0.0001	****		
8g	Young	VU0467154 / VU6028418 effects on gamma power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	32.63	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	15.38	<0.0001	****		
						Dose x Time	30, 390	9.318	<0.0001	****		
8h	Young	VU0467154 / VU6028418 effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	3, 39	2.125	0.1126	ns	13-14	Veh/Veh vs VU154 30mg/kg/Veh: 7hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0 and 2hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1and 2hrs
						Time	10, 130	33.58	<0.0001	****		
						Dose x Time	30, 339	4.471	<0.0001	****		
9a	Young	VU0467154 effects on delta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	47.32	<0.0001	****	14	1mg/kg vs Veh time: 1and 2hrs 3 mg/kg vs Veh time: 0, 1, 2, 3 and 5hrs 10 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5 and 6hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	23.75	<0.0001	****		
						Dose x Time	40, 520	5.798	<0.0001	****		
9b	Young	VU0467154 effects on theta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	4.257	0.0047	**	14	3 mg/kg vs Veh time: 1, 2 and 3hrs 10 mg/kg vs Veh time: 1, 2, 3, 4 and 5hrs 30 mg/kg vs Veh time: 0, 1, 2, 3, 4, 5, 7 and 8hrs
						Time	10, 130	41.10	<0.0001	****		
						Dose x Time	40, 520	3.352	<0.0001	****		
9c	Young	VU0467154 effects on alpha power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	22.97	<0.0001	****	14	1mg/kg vs Veh time: 1and 2hrs 3 mg/kg vs Veh time: 1, 2 and 5hrs 10 mg/kg vs Veh time: 1, 2, 3, 4, 5 and 6hrs 30 mg/kg vs Veh time: 1, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	28.76	<0.0001	****		
						Dose x Time	40, 520	4.553	<0.0001	****		
9d	Young	VU0467154 effects on beta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	6.621	0.0002	***	14	1mg/kg vs Veh time: 2hrs 3 mg/kg vs Veh time: 1hrs 10 mg/kg vs Veh time: 1, 2 and 3hrs 30 mg/kg vs Veh time: 1, 2, 3, 4, 5, 6 and 7hrs
						Time	10, 130	25.41	<0.0001	****		
						Dose x Time	40, 520	5.545	<0.0001	****		

9e	Young	VU0467154 / VU6028418 effects on wake delta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	24.74	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: 1and 2hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	21.63	<0.0001	****		
						Dose x Time	30, 390	6.593	<0.0001	****		
9f	Young	VU0467154 / VU6028418 effects on wake theta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	12.15	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	17.36	<0.0001	****		
						Dose x Time	30, 390	7.110	<0.0001	****		
9g	Young	VU0467154 / VU6028418 effects on wake alpha power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	12.23	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	20.90	<0.0001	****		
						Dose x Time	30, 390	3.233	<0.0001	****		
9h	Young	VU0467154 / VU6028418 effects on wake beta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	18.81	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: -1 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: -1 0, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 5, 6, 7 and 8hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1 2, 3, 4, 5, 6 and 7hrs
						Time	10, 130	27.84	<0.0001	****		
						Dose x Time	30, 390	4.927	<0.0001	****		
10a	Young	VU0467154 effects on delta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	47.32	<0.0001	****	14	1mg/kg vs Veh time: 1and 2hrs 3 mg/kg vs Veh time: 0, 1 2, 3 and 5hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5 and 6hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	23.75	<0.0001	****		
						Dose x Time	40, 520	5.798	<0.0001	****		
10b	Young	VU0467154 effects on theta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	4.257	0.0047	**	14	3 mg/kg vs Veh time: 1 2 and 3hrs 10 mg/kg vs Veh time: 1 2, 3, 4 and 5hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 7 and 8hrs
						Time	10, 130	41.10	<0.0001	****		
						Dose x Time	40, 520	3.352	<0.0001	****		
10c	Young	VU0467154 effects on alpha power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	22.97	<0.0001	****	14	1mg/kg vs Veh time: 1and 2hrs 3 mg/kg vs Veh time: 1 2 and 5hrs 10 mg/kg vs Veh time: 1 2, 3, 4, 5 and 6hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	28.76	<0.0001	****		
						Dose x Time	40, 520	4.553	<0.0001	****		
10d	Young	VU0467154 effects on beta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 52	6.621	0.0002	***	14	1mg/kg vs Veh time: 2hrs 3 mg/kg vs Veh time: 1hrs 10 mg/kg vs Veh time: 1 2 and 3hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6 and 7hrs
						Time	10, 130	25.41	<0.0001	****		
						Dose x Time	40, 520	5.545	<0.0001	****		
10e	Young	VU0467154 / VU6028418 effects on wake delta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	24.74	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: 1and 2hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	21.63	<0.0001	****		
						Dose x Time	30, 390	6.593	<0.0001	****		
10f	Young	VU0467154 / VU6028418 effects on wake theta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	12.15	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	17.36	<0.0001	****		
						Dose x Time	30, 390	7.110	<0.0001	****		
10g	Young	VU0467154 / VU6028418 effects on wake alpha power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	12.23	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 1hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 130	20.90	<0.0001	****		
						Dose x Time	30, 390	3.233	<0.0001	****		
10h	Young	VU0467154 / VU6028418 effects on wake beta power	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	3, 39	18.81	<0.0001	****	14	Veh/Veh vs VU154 30mg/kg/Veh: -1 0, 1 2, 3, 4, 5, 6, 7 and 8hrs Veh/Veh vs Veh/VU418 10 mg/kg: -1 0, 4, 5, 6, 7 and 8hrs Veh/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 5, 6, 7 and 8hrs VU154 30mg/kg/Veh vs VU154 30mg/kg/VU418 10 mg/kg: 0, 1 2, 3, 4, 5, 6 and 7hrs
						Time	10, 130	27.84	<0.0001	****		
						Dose x Time	30, 390	4.927	<0.0001	****		
11a	Comparison	Young vs aged time in wake	Duration (min/2hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 24	0.1583	0.2714	ns	13	None
						Time	11, 264	68.17	<0.0001	****		
						Age x Time	11, 264	1.731	0.0667	ns		
11b	Comparison	Young vs aged time in NREM	Duration (min/2hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 24	0.0003467	0.9853	ns	13	None
						Time	11, 264	64.00	<0.0001	****		
						Age x Time	11, 264	1.55	0.1123	ns		
11c	Comparison	Young vs aged time in REM	Duration (min/2hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 24	4.659	0.0411	*	13	Aged vs young time: ZT6
						Time	11, 264	36.30	<0.0001	****		
						Age x Time	11, 264	2.049	0.244	*		
11d	Comparison	Young vs aged time in wake	Duration (min/12hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 24	0.1583	0.2714	ns	13	None
						Time	1, 24	321.2	<0.0001	****		
						Age x Time	1, 24	2.558	0.01228	ns		
11e	Comparison	Young vs aged time in NREM	Duration (min/12hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 24	0.0003467	0.9853	ns	13	NA
						Time	1, 24	360.6	<0.0001	****		
						Age x Time	1, 24	1.872	0.1839	ns		
11f	Comparison	Young vs aged time in REM	Duration (min/12hr)	n/a	Repeated Measures Two-Way ANOVA	Age	1, 24	4.659	0.0411	*	13	Aged vs young time: ZT0-12
						Time	1, 24	85.87	<0.0001	****		
						Age x Time	1, 24	3.003	0.0959	ns		

12a	Comparison	Young vs aged wake qEEG	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 24	3.326	0.0807	ns	13	None
						Frequency	79, 1896	2.578	<0.0001	****		
						Age x Frequency	79, 1896	2.489	<0.0001	****		
12b	Comparison	Young vs aged NREM qEEG	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 24	0.1752	0.6793	ns	13	None
						Frequency	79, 1896	2.239	<0.0001	****		
						Age x Frequency	79, 1896	1.989	<0.0001	****		
12c	Comparison	Young vs aged REM qEEG	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 24	11.55	0.0024	**	13	Aged vs Young Freq: 45, 50-51, 53-54, 57-64 and 67Hz
						Frequency	79, 1896	3.691	<0.0001	****		
						Age x Frequency	79, 1896	3.887	<0.0001	****		
12d	Comparison	Young vs Aged gamma power wake	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 24	20.06	0.0002	***	13	Aged vs Young time: 0, 2, 3, 4, 5 and 7hr
						Time	10, 240	1.611	0.1039	ns		
						Age x Time	10, 240	1.611	0.1039	ns		
12e	Comparison	Young vs Aged NREM delta (SWA)	% change from young	Inactive	Repeated Measures Two-Way ANOVA	Age	1, 24	0.9262	0.3455	ns	13	None
						Time	10, 240	3.761	0.0001	***		
						Age x Time	10, 240	3.761	0.0001	***		
12f	Comparison	Young vs aged wake qEEG	% change from young	Active	Repeated Measures Two-Way ANOVA	Age	1, 24	2.428	0.1322	ns	13	Aged vs Young Freq: 0.5, 3-4, 6, 10, 42-43Hz
						Frequency	79, 1896	5.603	<0.0001	****		
						Age x Frequency	79, 1896	5.603	<0.0001	****		
12g	Comparison	Young vs aged NREM qEEG	% change from young	Active	Repeated Measures Two-Way ANOVA	Age	1, 24	0.3133	0.5821	ns	13	None
						Frequency	79, 1896	2.111	<0.0001	****		
						Age x Frequency	79, 1896	2.111	<0.0001	****		
12h	Comparison	Young vs Aged gamma power wake	% change from young	Active	Repeated Measures Two-Way ANOVA	Age	1, 24	7.644	0.0108	*	13	Aged vs Young time: -1and 1hr
						Time	10, 240	0.9831	0.4587	ns		
						Age x Time	10, 240	0.9831	0.4587	ns		
12i	Comparison	Young vs Aged NREM delta (SWA)	% change from young	Active	Repeated Measures Mixed-Effects Model (REML)	Age	1, 24	4.271	0.0497	*	13	None
						Time	10, 236	2.683	0.0040	**		
						Age x Time	10, 236	2.497	0.0073	**		
13a	Aged	VU0467154 effects on time in Wake	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	7.182	0.0001	***	13	1mg/kg vs Veh time: ZT2 3 mg/kg vs Veh time: ZT2, 6 and 12 10 mg/kg vs Veh time: ZT2 and 8 30 mg/kg vs Veh time: ZT2, 4 and 6
						Time	11, 132	82.79	<0.0001	****		
						Dose x Time	44, 528	1.828	0.0013	**		
13b	Aged	VU0467154 effects on time in NREM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	8.384	<0.0001	****	13	1mg/kg vs Veh time: ZT2 3 mg/kg vs Veh time: ZT2 and 6 10 mg/kg vs Veh time: ZT2, 4 and 8 30 mg/kg vs Veh time: ZT2, 4, 6 and 8
						Time	11, 132	86.32	<0.0001	****		
						Dose x Time	44, 528	2.619	<0.0001	****		
13c	Aged	VU0467154 effects on time in REM	Duration (min/2hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	8.851	<0.0001	****	13	1mg/kg vs Veh time: ZT18 3 mg/kg vs Veh time: ZT4 10 mg/kg vs Veh time: at ZT2, 4 and 6 30 mg/kg vs Veh time: ZT0, 2, 4, 6 and 8
						Time	11, 132	34.86	<0.0001	****		
						Dose x Time	44, 528	5.184	<0.0001	****		
13d	Aged	VU0467154 effects on time in wake	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	7.182	0.0001	***	13	3 mg/kg vs Veh time: ZT0-12 10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12
						Time	1, 12	370.2	<0.0001	****		
						Dose x Time	4, 48	3.551	0.0129	*		
13e	Aged	VU0467154 effects on time in NREM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	8.384	<0.0001	****	13	3 mg/kg vs Veh time: ZT0-12 10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12
						Time	1, 12	375.8	<0.0001	****		
						Dose x Time	4, 48	6.315	0.0004	***		
13f	Aged	VU0467154 effects on time in REM	Duration (min/12hr)	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	4.297	0.0044	**	13	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12
						Time	1, 12	154.4	<0.0001	****		
						Dose x Time	4, 48	11.56	<0.0001	****		
13g	Aged	VU0467154 effects on time in wake	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	22.86	<0.0001	****	13	3 mg/kg vs Veh time: ZT16 10 mg/kg vs Veh time: ZT12, 14, 16 and 18 30 mg/kg vs Veh time: ZT14, 16, 18 and 20
						Time	11, 132	49.17	<0.0001	****		
						Dose x Time	44, 528	5.587	<0.0001	****		
13h	Aged	VU0467154 effects on time in NREM	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	24.31	<0.0001	****	13	3 mg/kg vs Veh time: ZT16 10 mg/kg vs Veh time: ZT14, 16 and 18 30 mg/kg vs Veh time: ZT14, 16, 18 and 20
						Time	11, 132	42.94	<0.0001	****		
						Dose x Time	44, 528	5.836	<0.0001	****		
13i	Aged	VU0467154 effects on time in REM	Duration (min/2hr)	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	8.819	<0.0001	****	13	3 mg/kg vs Veh time: ZT16 10 mg/kg vs Veh time: ZT0,16 and 18 30 mg/kg vs Veh time: ZT0, 2 and 4
						Time	11, 132	94.45	<0.0001	****		
						Dose x Time	44, 528	2.379	<0.0001	****		
13j	Aged	VU0467154 effects on time in wake	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	22.86	<0.0001	****	13	10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24
						Time	1, 12	229.4	<0.0001	****		
						Dose x Time	4, 48	9.293	<0.0001	****		
13k	Aged	VU0467154 effects on time in NREM	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	24.31	<0.0001	****	13	10 mg/kg vs Veh time: ZT12-24 30 mg/kg vs Veh time: ZT12-24
						Time	1, 12	187.5	<0.0001	****		
						Dose x Time	4, 48	9.089	<0.0001	****		
13l	Aged	VU0467154 effects on time in REM	Duration (min/12hr)	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	8.819	<0.0001	****	13	10 mg/kg vs Veh time: ZT0-12 30 mg/kg vs Veh time: ZT0-12
						Time	1, 12	388.0	<0.0001	****		
						Dose x Time	4, 48	4.695	0.0028	**		

14a	Aged	VU0467154 effects on NREM Bout #	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 48	7.848	<0.0001	****	13	10 mg/kg vs Veh NREM bout number: P<0.0001 30 mg/kg vs Veh NREM bout number: P=0.0007
14b	Aged	VU0467154 effects on NREM Bout duration	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 48	28.73	<0.0001	****	13	3 mg/kg vs Veh NREM bout duration: P=0.0023 10 mg/kg vs Veh NREM bout duration: P=<0.0001 30 mg/kg vs Veh NREM bout duration: P=<0.0001
14c	Aged	VU0467154 effects on REM sleep latency	Direct comparison	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 48	3.592	0.0122	*	13	30 mg/kg vs Veh REM latency: P=0.0169
14d	Aged	VU0467154 effects on NREM Bout #	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	4, 48	3.905	0.0080	**	13	10 mg/kg vs Veh NREM bout number: P=0.0029 30 mg/kg vs Veh NREM bout number: P=0.0150
14e	Aged	VU0467154 effects on NREM Bout duration	Direct comparison	Active	Repeated Measures One-Way ANOVA	Dose	4, 48	39.73	<0.0001	****	13	3 mg/kg vs Veh NREM bout duration: p=0.0049 10 mg/kg vs Veh NREM bout duration: P<0.0001 30 mg/kg vs Veh NREM bout duration: P<0.0001
15a	Aged	VU0467154 effects on Wake qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	8.995	<0.0001	****	13	1mg/kg vs Veh Freq: 0.5-3, 8-17, 19, 21-22, 26-46, 52-61, 62-67, 71, 73-79Hz 3 mg/kg vs Veh Freq: 0.5-3, 5-6, 8-60, 62-77, 79Hz 10 mg/kg vs Veh Freq: 0.5-6, 9-79Hz 30 mg/kg vs Veh Freq: 0.5-1,3-6, 10-72 and 74-79Hz
						Frequency	79, 948	32.53	<0.0001	****		
						Dose x Frequency	316, 3792	3.644	<0.0001	****		
15b	Aged	VU0467154 effects on NREM qEEG	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	30.06	<0.0001	****	13	1mg/kg vs Veh Freq: 0.5-2, 11, 13-79Hz 3 mg/kg vs Veh Freq: 2-4, 7-8 and 10-79Hz 10 mg/kg vs Veh Freq: 0.5, 2-4 and 6-79Hz 30 mg/kg vs Veh Freq: 0.5-79Hz
						Frequency	79, 948	36.83	<0.0001	****		
						Dose x Frequency	316, 3792	9.035	<0.0001	****		
15c	Aged	VU0467154 effects on REM qEEG	% change from BL	Inactive	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 52	6.318	0.0003	***	7-13	1mg/kg vs Veh Freq: 35, 40, 45 and 53Hz 3 mg/kg vs Veh Freq: 2, 6-7, 24-25, 27-36, 38-40, 43-46, 48-54, 60-61 64, 67-68, 71-72, 74-72, 79Hz 10 mg/kg vs Veh Freq: 0.5, 2-4, 7, and 24-79Hz 30 mg/kg vs Veh Freq: 2-7, 10, 19-22, and 24-79Hz
						Frequency	79, 1027	52.23	<0.0001	****		
						Dose x Frequency	316, 2988	4.720	<0.0001	****		
15d	Aged	VU0467154 effects on gamma power during wake	% change from BL	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 48	13.07	<0.0001	****	13	1mg/kg vs Veh time: 0, 1, 5 and 7hrs 3 mg/kg vs Veh time: 0, 1and 4hrs 10 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	34.86	<0.0001	****		
						Dose x Time	40, 480	6.336	<0.0001	****		
15e	Aged	VU0467154 effects on NREM delta (SWA)	% change from BL	Inactive	Repeated Measures One-Way ANOVA	Dose	4, 52	5.596	0.0009	***	13	1mg/kg vs Veh time: -2, 0, 1and 3hrs 3 mg/kg vs Veh time: -2, 0 and 1hrs 10 mg/kg vs Veh time: -2, 0, 1 2, 3, 4 and 5hrs 30 mg/kg vs Veh time: 0, 1 2 and 3hrs
						Time	10, 130	113.6	<0.0001	****		
						Dose x Time	40, 520	4.029	<0.0001	****		
15f	Aged	VU0467154 effects on Wake qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	26.61	<0.0001	****	13	1mg/kg vs Veh Freq: 3-4Hz 3 mg/kg vs Veh Freq: 2-4 and 8-79Hz 10 mg/kg vs Veh Freq: 0.5-6 and 8-79Hz 30 mg/kg vs Veh Freq: 1-4, 6 and 8-79Hz
						Frequency	79, 948	92.85	<0.0001	****		
						Dose x Frequency	316, 3792	13.22	<0.0001	****		
15g	Aged	VU0467154 effects on NREM qEEG	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	26.09	<0.0001	****	13	1mg/kg vs Veh Freq: 0.5, 3, 13, 19-20, 23-26, 28-40, 42-43, 45, 47-56, 59, 61 63-65, 67 and 74-77Hz 3 mg/kg vs Veh Freq: 3 and 11-79Hz 10 mg/kg vs Veh Freq: 0.5-3, 7-8 and 11-79Hz 30 mg/kg vs Veh Freq: 2-4 and 6-79Hz
						Frequency	79, 748	59.26	<0.0001	****		
						Dose x Frequency	316, 3792	7.059	<0.0001	****		
15h	Aged	VU0467154 effects on gamma power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	59.17	<0.0001	****	13	1mg/kg vs Veh time: 0hrs 3 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6 and 8hrs 10 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	34.26	<0.0001	****		
						Dose x Time	40, 480	13.51	<0.0001	****		
15i	Aged	VU0467154 effects on NREM delta (SWA)	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	8.304	<0.0001	****	13	10 mg/kg vs Veh time: 0, 1 2, 3 and 4hrs 30 mg/kg vs Veh time: 0, 1 2, 3 and 5hrs
						Time	10, 120	27.78	<0.0001	****		
						Dose x Time	40, 472	3.448	<0.0001	****		
16a	Aged	VU0467154 effects on delta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	6.198	0.0004	***	14	1mg/kg vs Veh time: 1hrs 3 mg/kg vs Veh time: 1hrs 10 mg/kg vs Veh time:1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	29.98	<0.0001	****		
						Dose x Time	40, 480	5.077	<0.0001	****		
16b	Aged	VU0467154 effects on theta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	2.408	0.0622	ns	14	1mg/kg vs Veh time: 0, 1 2, 3 and 4hrs 3 mg/kg vs Veh time: 0, 1 2 and 3hrs 10 mg/kg vs Veh time: 0 hrs 30 mg/kg vs Veh time: 2 hrs
						Time	10, 120	3.332	0.0007	***		
						Dose x Time	40, 480	1.487	0.0310	*		
16c	Aged	VU0467154 effects on alpha power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	4.450	0.0039	**	14	1mg/kg vs Veh time: 1and 2hrs 3 mg/kg vs Veh time: 1 2, 3, 4 and 5hrs 10 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	21.98	<0.0001	****		
						Dose x Time	40, 480	4.301	<0.0001	****		
16d	Aged	VU0467154 effects on beta power during wake	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	32.23	<0.0001	****	14	1mg/kg vs Veh time: 1hrs 3 mg/kg vs Veh time: 1 2, 3, 4 and 5hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	31.60	<0.0001	****		
						Dose x Time	40, 480	11.01	<0.0001	****		

16e	Aged	VU0467154 effects on delta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	36.85	<0.0001	****	14	3 mg/kg vs Veh time: 0, 1 2, 3 and 4hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6 and 7hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	34.40	<0.0001	****		
						Dose x Time	40, 480	16.11	<0.0001	****		
16f	Aged	VU0467154 effects on theta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	4.394	0.0042	**	14	1mg/kg vs Veh time: 8hrs 3 mg/kg vs Veh time: 2 and 3hrs 30 mg/kg vs Veh time: 1 2, 3, 7 and 8hrs
						Time	10, 120	18.49	<0.0001	****		
						Dose x Time	40, 480	2.303	<0.0001	****		
16g	Aged	VU0467154 effects on alpha power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	32.33	<0.0001	****	14	1mg/kg vs Veh time: 2 and 3hrs 3 mg/kg vs Veh time: 0, 1 2, 3, 4 and 8hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	28.43	<0.0001	****		
						Dose x Time	40, 480	9.905	<0.0001	****		
16h	Aged	VU0467154 effects on beta power during wake	% change from BL	Active	Repeated Measures Two-Way ANOVA	Dose	4, 48	54.99	<0.0001	****	14	1mg/kg vs Veh time: 0, 1and 4hrs 3 mg/kg vs Veh time: 0, 1 2, 3 and 5hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5 and 6hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	54.15	<0.0001	****		
						Dose x Time	40, 480	14.03	<0.0001	****		
17a	Aged	VU0467154 effects on NREM theta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	4.448	0.0039	**	14	1mg/kg vs Veh time: -2, 0, 1hrs 3 mg/kg vs Veh time: -2, 0, 7 and 8hrs 10 mg/kg vs Veh time: -2, 0 and 7hrs 30 mg/kg vs Veh time: 0, 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	42.95	<0.0001	****		
						Dose x Time	40, 480	2.902	<0.0001	****		
17b	Aged	VU0467154 effects on NREM alpha power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	15.76	<0.0001	****	14	1mg/kg vs Veh time: 0hrs 3 mg/kg vs Veh time: 0, 1 2 and 3hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5 and 8hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	89.34	<0.0001	****		
						Dose x Time	40, 480	10.09	<0.0001	****		
17c	Aged	VU0467154 effects on NREM beta power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	23.93	<0.0001	****	14	1mg/kg vs Veh time: -2, 0, 1 2 and 3hrs 3 mg/kg vs Veh time: 0, 1 2 and 3hrs 10 mg/kg vs Veh time: -2, 0, 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	100.9	<0.0001	****		
						Dose x Time	40, 480	9.758	<0.0001	****		
17d	Aged	VU0467154 effects on NREM gamma power	% change from BL	Inactive	Repeated Measures Two-Way ANOVA	Dose	4, 48	14.75	<0.0001	****	14	1mg/kg vs Veh time: 0, 1and 3hrs 3 mg/kg vs Veh time: -2, 0, 1 2, 3, 4 and 6hrs 10 mg/kg vs Veh time: -2, 0, 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	17.52	<0.0001	****		
						Dose x Time	40, 480	4.400	<0.0001	****		
17e	Aged	VU0467154 effects on NREM theta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	3.355	0.0168	*	14	1mg/kg vs Veh time: 3hrs 10 mg/kg vs Veh time: 1 2, 3 and 4hrs 30 mg/kg vs Veh time: 2 and 3hrs
						Time	10, 120	17.51	<0.0001	****		
						Dose x Time	40, 472	1.917	0.0009	***		
17f	Aged	VU0467154 effects on NREM alpha power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	9.727	<0.0001	****	14	3 mg/kg vs Veh time: 1hrs 10 mg/kg vs Veh time: 0 and 1hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 7 and 8hrs
						Time	10, 120	40.67	<0.0001	****		
						Dose x Time	40, 472	5.430	<0.0001	****		
17g	Aged	VU0467154 effects on NREM beta power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	33.39	<0.0001	****	14	1mg/kg vs Veh time: 1 2 and 3hrs 3 mg/kg vs Veh time: 0, 1 2, 3 and 5hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	73.87	<0.0001	****		
						Dose x Time	40, 472	8.554	<0.0001	****		
17h	Aged	VU0467154 effects on NREM gamma power	% change from BL	Active	Repeated Measures Mixed-Effects Model (REML)	Dose	4, 48	18.37	<0.0001	****	14	1mg/kg vs Veh time: 3hrs 3 mg/kg vs Veh time: 1 2 and 3hrs 10 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs 30 mg/kg vs Veh time: 0, 1 2, 3, 4, 5, 6, 7 and 8hrs
						Time	10, 120	37.85	<0.0001	****		
						Dose x Time	40, 472	3.758	<0.0001	****		

Table 5.6. Detailed statistical analysis.

CHAPTER 6

Project Summary and Future Directions

6.1. Age-related Changes in Sleep/Wake Architecture and qEEG

In the present studies, several age-related changes in sleep-wake architecture and qEEG were observed. During the inactive phase 22-26-months-old mice displayed a pronounced reduction in REM sleep. This was replicated at 19-20-months-old, although with a lower magnitude of deficit. Consistent with these findings, reduced REM sleep is commonly seen during normal clinical aging (Ohayon et al., 2004).

When assessing NREM sleep during the inactive phase, 20-21-month-old mice displayed a trend towards the reduced duration of NREM sleep bouts, a measure of sleep fragmentation, compared to 4-5-month-old mice. In non-pathologically aged clinical populations, increased nighttime awakenings and sleep fragmentation are commonly seen (J. Li et al., 2018), consistent with the finding of reduced NREM sleep bout duration. Interestingly, no change in relative delta power (SWA) during NREM sleep was observed. These changes are similar to published data which showed non-pathologically aged mice had increased absolute delta power from 2Hz when compared to young mice (McKillop et al., 2018; Panagiotou et al., 2017). This differs from clinical populations who exhibit reduced delta power (SWA) during NREM sleep (Carrier et al., 2001; Darchia et al., 2007; Yoon et al., 2021).

During the active phase, non-pathologically aged mice from 19-months-old displayed reduced wakefulness and increased wake fragmentation, consistent with clinically described daytime napping observed in increased age (J. Li et al., 2018). During waking epochs during the active phase, non-pathologically aged mice displayed reduced arousal with decreased gamma power. Additionally, decreased alpha power and increased delta power were seen indicating shifts from high to low frequencies. These changes are consistent with reduced arousal and shifts to

lower EEG frequencies observed in non-pathological aging and patients with AD (D'Atri et al., 2021; Meghdadi et al., 2021; Murty et al., 2020; Vecchio et al., 2013).

6.2. Direct and Indirect Acting Muscarinic Agonists

In the present work, I compared the effects of the direct-acting M_1/M_4-preferring muscarinic agonist xanomeline and the indirect-acting muscarinic agonist donepezil. Donepezil is an AChEI which prevents the synaptic breakdown of acetylcholine and represents the current standard of care for treating cognitive deficits in AD (Sharma, 2019). Interestingly, donepezil increased wakefulness and arousal in young mice during the inactive phase, but not the active phase, and displayed an impact on wake or arousal in non-pathologically aged mice during either the inactive or active phase. In contrast, the direct-acting M_1/M_4 preferring muscarinic agonist xanomeline produced increased wakefulness and arousal during the inactive phase in young mice, and during both the inactive and active phase in non-pathologically aged mice.

These data highlight the potentially increased efficacy of direct-acting muscarinic activators rather than indirect-acting agonists in treating populations with reduced central cholinergic integrity. Alternative methods for directly targeting mAChRs have included the development of PAMs which produce increased selectively compared to orthosteric ligands (Conn et al., 2009; C. K. Jones et al., 2012). Furthermore, these data following xanomeline and donepezil dosing highlight the differences in efficacy observed when a cholinergic compound is administered at different circadian time points. Both donepezil and xanomeline consistently reduced delta power (SWA) during NREM sleep in young and non-pathologically aged mice, suggesting the potential to disrupt cognition or increase AD pathology (C. Wang and Holtzman, 2020). As such, when investigating the contribution of M_1 and M_4 mAChRs on the modulation of sleep/wake architecture and arousal utilizing more selective ligands, it is important to assess not just effects in aging, but also effects across the circadian rhythm.

6.3. Circadian and Aging Effects on Cholinergic Modulation

The effects of PAMs are known to be dependent on the degree of endogenous signaling (Conn et al., 2009; C. K. Jones et al., 2012), and central cholinergic structure and/or signaling has been reported to decline with increasing age in both clinical populations (Kanel et al., 2022; Richter et al., 2014) and in preclinical species (Casu et al., 2002; Fischer et al., 1992; Mitsushima et al., 1996; Xie et al., 2019). Given the reported decreases in central cholinergic structure and signaling with age, the efficacy of mAChR PAMs would be expected to differ between young and non-pathologically aged animals. Furthermore, circadian-dependent fluctuations in cholinergic modulation have been well described, with levels of acetylcholine being highest during the active phase (light's off for rodents) and lowest during the inactive phase (light's on for rodents) (Mitsushima et al., 1996). As such, it stands to reason that the efficacy of PAMs targeting mAChRs will be dependent on whether dosing occurs during the inactive or active period.

In the described studies, the effects of both the M_1 mAChR PAM VU0453595 and the M_4 mAChR PAM VU0467154 on sleep/wake architecture and qEEG are examined. Initial experiments explored dosing with the M_1 mAChR PAM VU0453595 in the inactive phase in young mice and rats, and dosing during the active phase in non-human primates (NHPs). As M_1 mAChR PAMs were hypothesized to be wake-promoting, inactive phase dosing was performed in rodents as there would be reduced wake and arousal allowing a sufficient window to observe wake and arousal-promoting effects. NHPs display few wakening's during the night, so active phase dosing was necessary to assess effects on arousal during wake epochs. Increased wakefulness and increased high gamma during wakefulness were observed in mice and rats, and increased alpha and gamma powers during wake in NHPs. In non-pathologically aged (22-26-month-old) mice, VU0453595 enhanced wakefulness when dosed in the inactive phase, however the magnitude of the gamma power increases during waking epochs was reduced compared to young (3-5-month-old) mice. I hypothesized that this reduction in efficacy was due to a reduction in central

cholinergic structure and signaling, meaning a reduced concentration of endogenous acetylcholine to act in conjunction with the M_1 mAChR PAM VU0453595.

I further report dosing M_1 and M_4 mAChR PAMs across the circadian rhythm in young (3-5-month-old) and non-pathologically aged (19-21-month-old or 26-28-month-old) mice. As the M_1 PAM VU0453595 was previously observed to have reduced efficacy in the inactive phase in 22-26-month-old mice I hypothesized that a greater effect might be observed in the active phase when there are higher levels of acetylcholine. Further, if M_1 mAChR PAMs can normalize disruptions in wake and arousal in the active phase this could suggest a benefit in clinical populations when dosing during the daytime. Initially, studies assessed the effects of the M_1 mAChR PAM VU0453595 in 3-4-month-old mice when dosed during the inactive and active phases. In the inactive phase the previously described increases in wakefulness and gamma power were replicated. In contrast, during the active phase there was no effect on wake in 3-4-month-old mice and only a modest effect on gamma power during wake. In contrast, in 19-20-month-old mice VU0453595 dosing increased wakefulness and gamma power during wake following inactive or active phase dosing, suggesting M_1 mAChR PAMs normalize the reduced arousal seen during the active phase in aged mice.

Dosing 26-28-month-old mice with the M_1 mAChR PAM VU0453595 in the active phase was performed to test the hypothesis that the magnitude of gamma power increases would be greater when dosing in the active phase than the previously observed effects in the inactive phase in 22-26-month-old mice. Dosing in the active phase produced a robust increase in wakefulness, however only modest increases in gamma power; similar to previously described inactive phase studies in 22-26-month-old mice. One hypothesis for this reduced effect size is age-related decreased concentration of the endogenous ligand, acetylcholine. To test this, the M_1 mAChR PAM VU0453595 was co-dosed with the acetylcholinesterase inhibitor donepezil which produced a larger increase in gamma power, and a wake qEEG profile similar to what was observed with xanomeline. These data suggest that in clinical populations with large reductions in cholinergic

signaling, a combination of an AChEI and an M_1 mAChR PAM may provide increased enhancement of wake and arousal over either compound alone. Importantly, when dosed alone in the inactive phase VU0453595 had no effect on delta power (SWA) in non-pathologically aged mice suggesting VU0453595 would not disrupt NREM sleep quality in the same manner as xanomeline and donepezil.

Modulation at the M_4 mAChR receptor with the M_4 PAM VU0467154 produced increased NREM sleep in both phases in young and non-pathologically aged mice, and reduced REM sleep when dosed in the inactive phase. In the inactive phase increased NREM sleep bout length was observed with concurrent reductions in bout number, indicating reduced sleep fragmentation, suggesting potential clinical efficacy in normalizing age- and AD-related sleep deficits. When dosed in the inactive phase VU0467154 produced moderately increased delta power (SWA) during NREM sleep in young animals, with a greater magnitude observed in 20-21-month-old animals. This increase in delta power (SWA) during NREM sleep suggests a possible sleep-dependent mechanism through which M_4 mAChR PAMs may enhance cognition and reduce AD pathology.

6.3.1. Aging Effect on Central Cholinergic Structure

As I hypothesized that differences in compound efficacy with dosing were due to changes in central cholinergic integrity, in the current data I have assessed the effects of aging on cholinergic terminal density in the PFC. The basal cholinergic forebrain, which projects to the PFC, has been shown to display age-related changes in cholinergic cell bodies (Fischer et al., 1992; Gibbs, 1998), and the PFC is vitally important for the control of wake and arousal (B. E. Jones, 2020). Interestingly, this dataset displayed no age-related change in PFC cholinergic fiber density. Previous studies utilizing ChAT IHC in aged mice have demonstrated reductions in ChAT-positive fiber density in hippocampal and parietal cortical regions, potentially suggesting regional-specific reductions in cholinergic structural integrity (Xie et al., 2019). Future studies will

		Compound	Mechanism	Wake	NREM sleep	REM sleep	Sleep/Wake Fragmentation	Arousal during wake	NREM sleep quality
Young (3-5-months)	Active	Donepezil	AChEI	Increased	Decreased	No effect	No effect	No effect	No effect
		Xanomeline	M_1/M_4-preferring orthosteric agonist	Increased	Decreased	Increased	No effect	No effect	Decreased
		VU0453595	M_1 mAChR PAM	No effect	No effect	No effect	No effect	Increased	No effect
		VU0467154	M_4 mAChR PAM	Decreased	Increased	Increased	No effect	Decreased	No effect
	Inactive	Donepezil	AChEI	Increased	Decreased	Decreased	Decreased	Increased	Decreased
		Xanomeline	M_1/M_4-preferring orthosteric agonist	Increased	Decreased	Decreased	No effect	Increased	Decreased
		VU0453595	M_1 mAChR PAM	Increased	Decreased	No effect	No effect	Increased	Decreased
		VU0467154	M_4 mAChR PAM	Decreased	Increased	Decreased	Decreased	Decreased	Increased
Aged (19-21-months)	Active	Donepezil	AChEI	No effect	No effect	Increased	No effect	No effect	No effect
		Xanomeline	M_1/M_4-preferring orthosteric agonist	Increased	Decreased	No effect	Decreased	Decreased	Decreased
		VU0453595	M_1 mAChR PAM	Increased	Decreased	No effect	No effect	Increased	Decreased
		VU0467154	M_4 mAChR PAM	Decreased	Increased	Increased	Decreased	No effect	Increased
	Inactive	Donepezil	AChEI	Increased	Decreased	Decreased	No effect	Increased	Decreased
		Xanomeline	M_1/M_4-preferring orthosteric agonist	Increased	Decreased	Decreased	No effect	Increased	Decreased
		VU0453595	M_1 mAChR PAM	Increased	Decreased	No effect	Increased	Increased	No effect
		VU0467154	M_4 mAChR PAM	Decreased	Increased	Decreased	Decreased	Decreased	Increased
>24-months	Active	VU0453595	M_1 mAChR PAM	Increased	Decreased	No effect	No effect	Increased	No effect
		VU0453595 + Donepezil	M_1 mAChR PAM + AChEI	Increased	Decreased	Increased	Decreased	Increased	Decreased
	Inactive	VU0453595	M_1 mAChR PAM	Increased	Decreased	Increased	Not assessed	Increased	Not assessed
		VU0453595 + Donepezil	M_1 mAChR PAM + AChEI	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested

Table 6.1. Summary of compound effects on sleep/wake architecture, arousal and NREM sleep quality

be needed to assess if this finding replicates. The lack of age-related change in PFC cholinergic structural integrity in the present study may be a consequence of the method utilized to assess central cholinergic structures. Previous studies have detected age-related decreases in cholinergic boutons in layer V of the PFC in aged rats (Casu et al., 2002), however the immunohistochemistry methodology utilized does not have sufficient resolution to detect changes in boutons. Alternatively, there may be no structural change but a functional change. Previous

studies have indicated that rats display age-related decreases in circadian ACh fluctuations and reduced ACh levels when measured by microdialysis in the PFC (Mitsushima et al., 1996; C. F. Wu et al., 1988). It is possible that while the neuronal structure is preserved there is a reduction in ACh release.

6.4. Summary of Cholinergic Ligand Effects and Implications for Cognition and AD pathology

The effects of the tested cholinergic compounds on numerous sleep/wake architecture and arousal endpoints vary depending on circadian timepoint and age at dosing (see Table 6.1 for summary). These observed effects have important implications for predicted impact on cognitive functioning and AD disease pathology. Generally, increased wakefulness and arousal with reduced wake fragmentation during the active phase normalize deficits in non-pathologically aged mice and would be predicted to result in increased cognitive function. In contrast, during the inactive phase increased NREM sleep and NREM sleep quality during the inactive phase, when sleep is predominant would be hypothesized to support cognitive performance and potentially decrease AD pathology (C. Wang and Holtzman, 2020).

From the data presented I can hypothesize the potential clinical implications for each cholinergic ligand tested (see Figure 6.1. for summary). Donepezil produced no effect on deficits in wake and arousal when in the active phase, so no positive effect on cognition would be expected. Furthermore, inactive phase dosing with donepezil produced decreased NREM and REM sleep duration, and decreased NREM sleep quality. These effects on sleep would be hypothesized to worsen cognitive performance and potentially increase AD pathology. In contrast, xanomeline normalized wake and arousal deficits in non-pathologically aged mice, effects that predict improvements in cognitive performance. However, similar to donepezil, xanomeline decreased NREM sleep, REM sleep and NREM sleep quality, again suggesting a possible contraindication to nighttime dosing in clinical populations with the potential to decrease cognitive

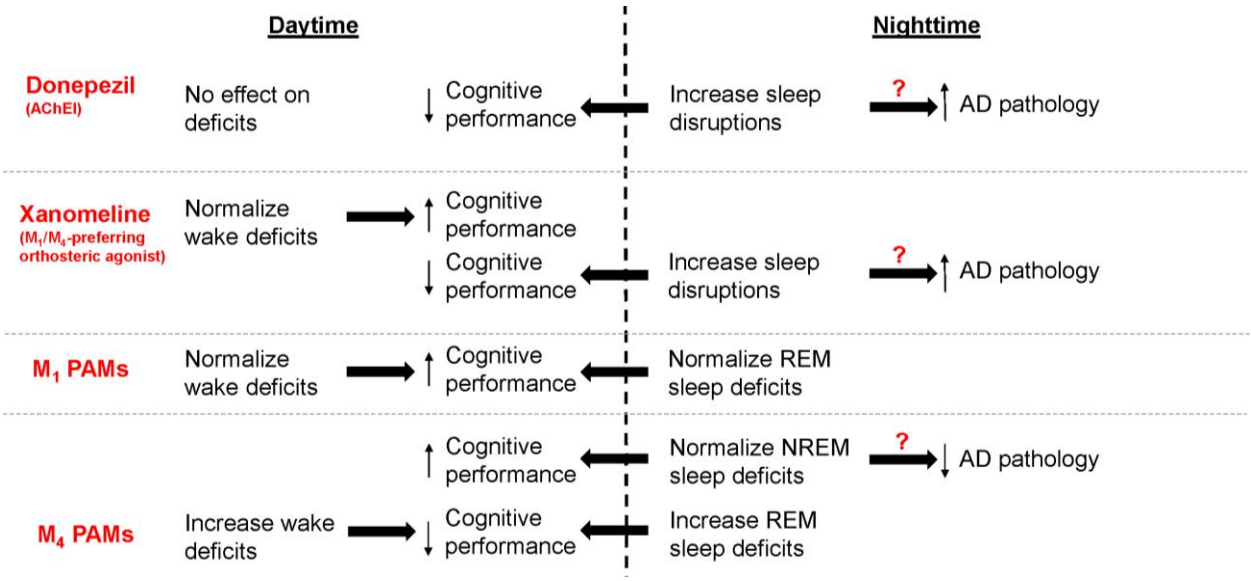

Figure 6.1. Summary of hypothesized cholinergic compound effects on cognitive performance and AD pathology

performance and an increase in AD pathology. The M_1 mAChR PAM, VU0453595, normalized wake and arousal deficits in non-pathological aging, predicting increased cognitive performance, and did not reduce NREM sleep quality during the inactive phase. Furthermore, VU0453595 normalized REM sleep deficits in 22-26-month-old mice that displayed more severe REM sleep deficits. Taken together, this suggests that dosing during sleep may produce a subsequent improvement in cognitive performance through REM sleep enhancement and importantly will not have the potential to worsen AD pathology. The M_4 mAChR PAM, VU0467154, reduced wake and decreased arousal when dosed during the active phase, suggesting M_4 activation during wake may impair cognitive performance. In contrast, inactive phase dosing reduced NREM sleep fragmentation, and increased NREM sleep quantity and quality, which may not only improve subsequent cognition, but also reduce AD pathology through the enhancement of glymphatic clearance (C. Wang and Holtzman, 2020).

Finally, it is important to consider the effects of improved sleep or wake on subsequent circadian periods. For example, increasing nighttime NREM sleep and NREM sleep quality may support increased wakefulness and arousal on subsequent days. Conversely, increased daytime

wake and arousal, with reduced daytime napping may result in increased sleep duration and NREM sleep quality the following night. The present studies have assessed the immediate effects of compound dosing on qEEG parameters. Future studies will be able to determine the potential carry-over effects of these compounds and further improve our understanding of the potential clinical implications of these compounds.

6.5. M_1 mAChR PAM Indications in Alzheimer's Disease Populations

In the present studies, I have demonstrated that the M_1 mAChR PAM VU0453595 can enhance wakefulness, and gamma power, a correlate of cognition and arousal (Buzsáki and Silva, 2012), translating from mice to non-human primates. These data were extended with an assessment of the M_1 mAChR PAM VU0453595 in young and non-pathologically aged mice across circadian phase. The findings of these studies suggest that M_1 PAMs dosed in clinical populations during the active phase would produce wake and arousal-promoting effects. However, in cases of more advanced disease cholinergic degeneration would be expected to be more marked and the M_1 mAChR PAM effects on arousal may be attenuated. The findings shown, suggest that M_1 mAChR PAM effects are enhanced by a subthreshold dose of the AChEI donepezil, implying that in populations with more severe cholinergic degeneration, an AChEI may be used to enhance the existing cholinergic tone and allow improved efficacy of an M_1 mAChR PAM.

Whether dosing an M_1 mAChR PAM during the inactive phase, i.e., prior to sleeping, has benefit in AD is less clear. Doses that produce wakefulness in aged animals in the active phase also do so during the inactive phase, suggesting these doses may be disruptive to sleep, or at least increase sleep latency. I demonstrated that the M_1 mAChR PAM does not impact delta power (SWA) during NREM, whereas acetylcholinesterase inhibitors reduce delta power (SWA) during NREM sleep, indicating NREM sleep disruption. Furthermore, the M_1 mAChR PAM

VU0453595 normalized REM sleep deficits in non-pathologically aged mice with more marked REM sleep deficits. It is important to note that AChEIs have been suggested to increase REM sleep in clinical populations (Moraes et al., 2006), and it has been suggested that this leads to nightmares reported as a side effect in some patients (Dunn et al., 2000; Ridha et al., 2018). To date, no studies have related the loss of REM sleep in aging and AD with symptomatology, cognitive dysfunction, or pathology. However, REM sleep is implicated in emotional memory, emotional regulation, spatial memory, and motor memory (Peever and Fuller, 2017), so it stands to reason that normalizing observed reductions in REM sleep may improve deficits in these cognitive functions in aging and AD.

6.6. M_4 mAChR PAM Indication in Schizophrenia and Alzheimer's Disease Populations

The M_4 mAChR PAM has been demonstrated to promote NREM sleep and suppress REM sleep in young and non-pathologically aged mice. This increased NREM sleep is seen with increased NREM sleep bout length and reduced NREM sleep bout number, reducing fragmentation during NREM sleep. AD patients display increased fragmentation and reduced NREM sleep (Bubu et al., 2017; Peter-Derex et al., 2015; Prinz, Vitaliano, et al., 1982), suggesting these effects would be beneficial in clinical populations. However, as previously discussed, AD patients display reduced REM sleep, and so further decreasing this may exacerbate clinical symptoms. The exception to this could be patients experiencing sleep disturbances such as nighttime awakenings and nightmares due to existing AChEI treatment (Dunn et al., 2000; Ridha et al., 2018), in these cases M_4 mAChR PAMs could prove to be a beneficial adjunct therapy. In schizophrenia, patients display reduced REM sleep latency, which has been correlated with decreased cognitive performance (Das et al., 2005; Ferrarelli, 2021), as such the observed decreased REM sleep with increased REM sleep latency with the M_4 mAChR PAM VU0467154 would normalize the observed sleep deficit and may produce further symptomatic improvement.

During wake, NREM sleep, and REM sleep VU0467154 produced shifts from higher to lower frequency EEG. During NREM sleep increased NREM sleep quality, as measured by delta power (SWA), is observed, which is disrupted in both AD (Y. Zhang et al., 2022) and schizophrenia (Kaskie et al., 2019). This suggests potential benefit in normalizing these abnormalities in clinical populations. The EEG slowing observed during wake following dosing with the M_4 mAChR PAM VU0467154 would exacerbate EEG slowing already present in AD populations which correlates with cognitive decline (Cecchetti et al., 2021; Claus et al., 1998). In contrast, in schizophrenic patients experiencing psychosis display increased gamma powers (Baldeweg et al., 1998; Yadav et al., 2021), suggesting that these effects may be beneficial for the treatment of psychosis.

6.7. Future Directions

6.7.1. Combination Studies

When assessing potential treatments for AD it is important to remember that most patients experiencing MCI and AD will already be receiving treatment, most commonly with AChEIs. As such it is important to understand how these compounds interact with AChEIs. Initial studies utilizing acute dosing paradigms in mice have been performed with the M_1 mAChR PAM VU0453595, demonstrating that VU053595 enhances the effects of a subthreshold, acute dose of donepezil. Future studies will be needed to understand the effects of chronic AChEI administration on sleep-wake architecture in non-pathologically aged mice, and then how both acute and chronic administration of M_1 and M_4 mAChR PAMs in the presence of a chronically dosed AChEI further modify sleep-wake architecture and arousal.

6.7.2. Assessing Effects of M_4 PAMs on Glymphatic Clearance and Disease Modification

Recent work has suggested that modulation of NREM sleep may be a viable target for disease modification in patients with AD (Y. F. Lee et al., 2020). Numerous studies have identified associations between quantifiably poorer sleep (duration, fragmentation, reduced sleep quality) and AD pathology (Lucey et al., 2019; Spira et al., 2013), with an increasing number of studies attempting to identify the directionality of this association (C. Wang and Holtzman, 2020). Recent studies have suggested that decreased NREM sleep quantity and quality leads to increased AD pathology (Shokri-Kojori et al., 2018; Winer et al., 2020). It has been suggested that glymphatic clearance is important for the clearance of amyloid and tau pathology preclinically (Iliff et al., 2014; Peng et al., 2016), and that glymphatic clearance increases during NREM sleep (Mendelsohn and Larrick, 2013). Studies have suggested that increased delta power (SWA) during NREM sleep is coupled with glymphatic activity (Fultz et al., 2019), as such, with the M_4 mAChR PAM VU0467154 increasing NREM sleep duration and delta power (SWA) during NREM sleep it is possible that M_4 mAChR PAMs may reduce AD pathology by enhancing glymphatic clearance.

Future studies will be needed to assess the effects of M_4 mAChR PAMs on glymphatic activity in rodents by assessing the dynamic cerebrospinal-interstitial fluid (CSF-ISF) exchange, using MRI contrast administration into the intra-cisternal followed by MRIs with and without an M_4 mAChR PAM. Further studies could also investigate the effects of an M_4 mAChR PAM chronically dosed on pathology in a mouse model of AD.

6.7.3. Further Assessment of Anatomical and Signaling Changes Observed in Aging

The current study has assessed the structural changes observed in the cholinergic system in the PFC with non-pathological aging. This area was assessed due to high levels of cholinergic innervation (van de Werd et al., 2010), with many of these projections arising from the basal cholinergic forebrain (Ährlund-Richter et al., 2019; M. M. Mesulam, 1990; M. -Marsel Mesulam et al., 1983), an area known to degenerate in AD (Whitehouse, Price, Struble, Clark, Coyle, and

DeLong, 1982). While the present study found no changes in cholinergic fiber density, a recent study utilizing similar immunohistochemical methods in non-pathologically aged C57Bl/6J mice revealed reduced central cholinergic fiber density in the dorsal hippocampus and parietal cortex (Xie et al., 2019). Future studies will aim to assess the cholinergic innervation of the dorsal and ventral hippocampus, with the dorsal hippocampus hypothesized to be crucial in cognition and the ventral hippocampus in the regulation of stress, emotion, and affect (Fanselow and Dong, 2010). Cognition and regulation of stress, emotion and affect are known to be cholinergic-dependent, and display alterations with non-pathological aging (Drachman and Leavitt, 1974; Janowsky et al., 1972; Kessler and Staudinger, 2009).

Beyond understanding the effects of non-pathological aging on central cholinergic integrity, it is important to understand how receptor levels may change with aging to fully understand the mechanism underlying the differences in compound effects. As such, future studies will use RNAscope and/or radioligand binding methods to compare M_1 and M_4 mAChR RNA and/or receptor levels in young and non-pathologically aged mice.

APPENDICES

Appendix A: The Effects of Aging and the M_1 PAMs VU0486846, and VU0453595, and Donepezil on Cognition

Introduction

M_1 mAChR PAMs have previously been demonstrated to enhance cognition in several hippocampal-dependent tasks including novel object recognition (NOR) (Moran et al., 2018; Rook et al., 2018) and a touchscreen pairwise discrimination task (Gould et al., 2015). To date, all studies have been performed in young animals during the inactive phase, when cholinergic signaling is low (Mitsushima et al., 1996). As M_1 PAM efficacy is dependent on existing cholinergic signaling, it will be important to understand the cognitive enhancing properties of M_1 PAMs both in the active phase and in aged animals. In this section, I will discuss data assessing circadian differences in the efficacy of the M_1 mAChR PAM VU0486846 in the NOR task, the efficacy of the M_1 mAChR PAM VU0486846 in a touchscreen pairwise discrimination task, compare the acquisition curves of young and aged mice in a touchscreen pairwise discrimination task and assess the efficacy of the M_1 PAM VU0453595 in aged animals in a touchscreen pairwise discrimination task.

Methods

Novel object recognition (NOR)

8–12-week-old male C57BL/6 mice (n=294, Taconic) were habituated to the test chamber and intraperitoneal injection 24-hrs before testing. Mice were administered VU0486846 or vehicle (10% tween 80) and donepezil or vehicle (saline) via intraperitoneal injection 30 minutes prior to exposure to 2 identical objects for 10 minutes. 24-hrs later mice were exposed to one familiar and

one novel object for 5 minutes. Blinded observers scored the duration of time mice explored each object and Discrimination Index was calculated (DI= time exploring novel – time exploring familiar object/total exploration time).

Pairwise discrimination

Young (3-month-old, Taconic, n=20) and aged (18-month-old, Jackson Laboratories, n=13) C57/b6 mice were pair housed and weighed at baseline. Food restriction was initiated over one week to lower animals to 85-90% of free-feeding bodyweight with weights recorded daily. During this period 30% original (3-month-old) or strawberry (18-month-old) ensure in water was placed in the mice's home cage to allow them to habituate to the food reward mice then progressed through 5 stages of touchscreen training. Throughout training and testing a 2-hole mask was placed over the touchscreen so responses could only be made where images were displayed.

Stage 1: Animals were habituated to the chamber and received diluted Ensure (30µl) delivered by a peristaltic pump into a receptacle opposite the touch screen. Once animals were consistently consuming liquid rewards (30 rewards inside 30 minutes) they progressed to stage 2.

Stage 2: A stimulus was presented on the screen for 3 seconds, following which a liquid Ensure reward would be delivered. Animals would progress when 30 rewards were consumed in 30 minutes)

Stage 3: As with stage 2 a stimulus was presented on the screen, here the mouse was required to nose poke the touchscreen (breaking the infrared beam) to trigger the reward delivery. The criteria to progress was again to complete 30 trials in 30 minutes.

Stage 4: As with stage 3 except the mice had to initiate the trial by nose poking in the reward receptacle. Following the initiation of the trial the stimuli were presented on the screen and following a nose poke at one of the two stimuli the ensure reward would be delivered. The criteria to progress was again to complete 30 trials in 30 minutes.

Stage 5: The mice would trigger the trial as described in stage 4, then an image was displayed in one of the two locations and the mice were required to select the image to receive the liquid reward. Selection of the blank window was considered an incorrect response, terminating the trial resulting in the house light being extinguished for 5 seconds. Animals were required to complete 50 trials with an accuracy of ≥80% for 2 consecutive sessions within 1-hr before they were considered trained.

Following this the mice would progress to the pairwise discrimination task where the fan (S+) and marbles (S-) stimuli were used as this top-down version of the task has previously been demonstrated to be sensitive to M_1 modulation (Gould et al., 2015).

Young mouse studies

Animals were dosed with VU0486846 (3 mg/kg) or vehicle (10% tween 80) at 10ml/kg via intraperitoneal injection 30 minutes prior to the initiation of the task. During the initial acquisition of the task there were numerous technical difficulties with equipment malfunction so dosing was stopped after 5 days and animals were trained to criteria (2 sessions ≥80%). Following this a reversal learning paradigm was performed where the marbles became the rewarded stimuli (S+) and the fan the incorrect stimuli (S-). Performance was followed over 14-sessions.

Aged mouse studies

Animals were dosed with VU0453595 (3 mg/kg) or vehicle (5% BCD) at 10ml/kg via intraperitoneal injection 30 minutes prior to the initiation of the task. Performance was followed over 22-sessions.

In both cases mice received 60 trials in a 1-hr session and were required to achieve ≥80% accuracy with >50 trials completed for 2 consecutive sessions to reach the criteria). To be included in the final analysis mice had to complete ≥20/60 trials in a given session.

Statistics

For novel object recognition a one-way ANOVA with no repeated measures and a Dunnett's multiple comparisons was used if indicated. For touchscreen pairwise discrimination a

mixed effects model was used to compare acquisition curves and Log-Rank (Mantel-Cox) tests to compare survival plots.

Results

NOR in young mice

The M_1 mAChR positive allosteric modulator VU0486846 improved discrimination index when dosed in the inactive phase (p=0.0105, $F_{(3,40)}$=4.269), with a significant increase in discrimination index seen at 3 mg/kg (p=0.0041) (Figure A.1A). Donepezil dosed during the active phase produced a visual trend towards an increase in discrimination index, with maximal effect size at 0.01 mg/kg, however significance was not reached (p=0.203, $F_{(4,55)}$=1.542) (Figure A.1B). When VU0486846 was dosed in combination with a subthreshold dose of donepezil (0.003 mg/kg)

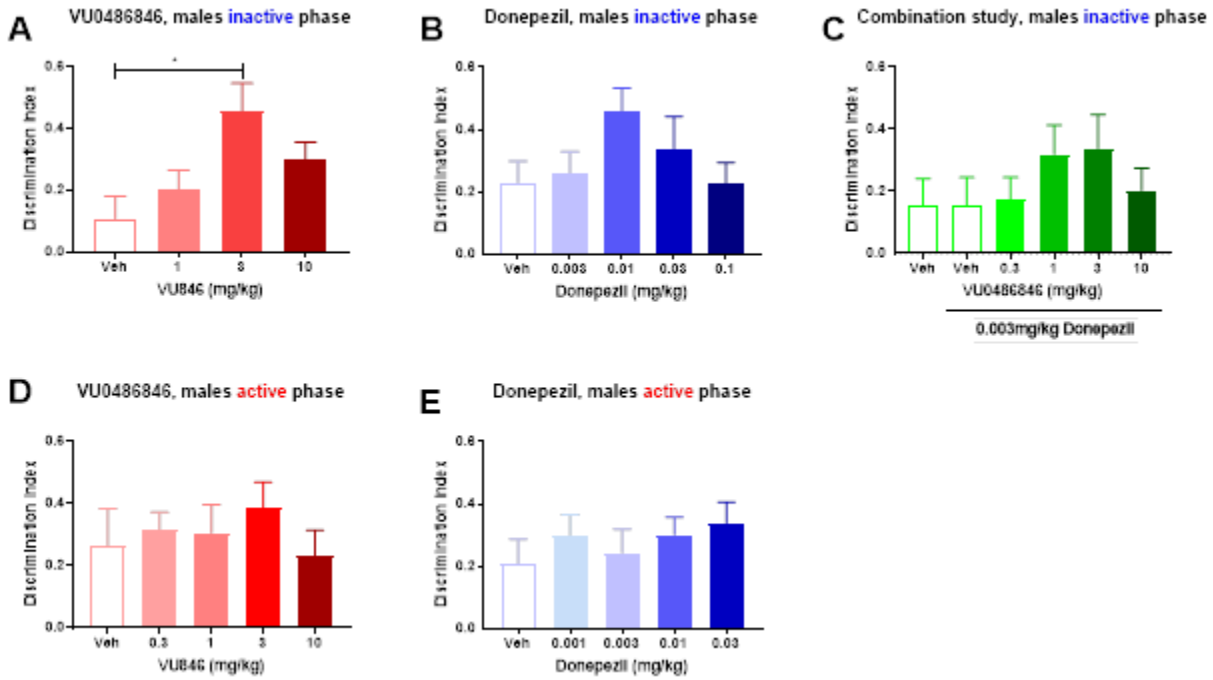

Figure A.1. VU0486846 enhances discrimination index during the inactive phase. Shown is the discrimination index in the novel object recognition task following dosing with VU0486846 (A), donepezil (B) and 0.003 mg/kg donepezil with VU0486846 (C) in the inactive phase, and VU0486846 (D) and donepezil (E) in the active phase. * indicates p<0.05, one way ANOVA with Dunnett's multiple comparisons. N=7-14 /group

no significant effect was seen (p=0.4827, $F_{(5,77)}$=0.9884) (Figure A.1C). When dosed in the active phase, neither VU0486846 (p=0.7828, $F_{(4,34)}$=0.4344) nor donepezil (p=0.6968, $F_{(4, 63)}$=0.05540) produced enhancement of the discrimination index (Figure A.1D and E)

Pairwise discrimination in young and aged mice

20 young (3-month-old) animals started initial training, however only 13 of these reached the criteria on the initial pairwise task prior to reversal learning. The M_1 PAM VU0486846 dosed at 3 mg/kg displayed no main effect of dose (p=0.6081, $F_{(1,6)}$=0.2925) or dose x time interaction (p=0.5565, $F_{(13, 58)}$=0.9014) on acquisition. A main effect of time was observed (p<0.0001, $F_{(13,78)}$=32.80) (Figure A.2A). No effect of dose on the percentage of animals reaching criteria at a given session was observed (p=0.3036, χ^2 = 1.058, df = 1) (Figure A.2B) on reversal learning of a pairwise discrimination task.

13 aged animals (18-month-old) started training however 3 were removed due to failure to complete the initial training phases. During the pairwise discrimination task the M_1 PAM VU0453595 dosed at 3 mg/kg displayed no main effect of dose (p=0.5036, $F_{(1,8)}$=0.4904) or dose

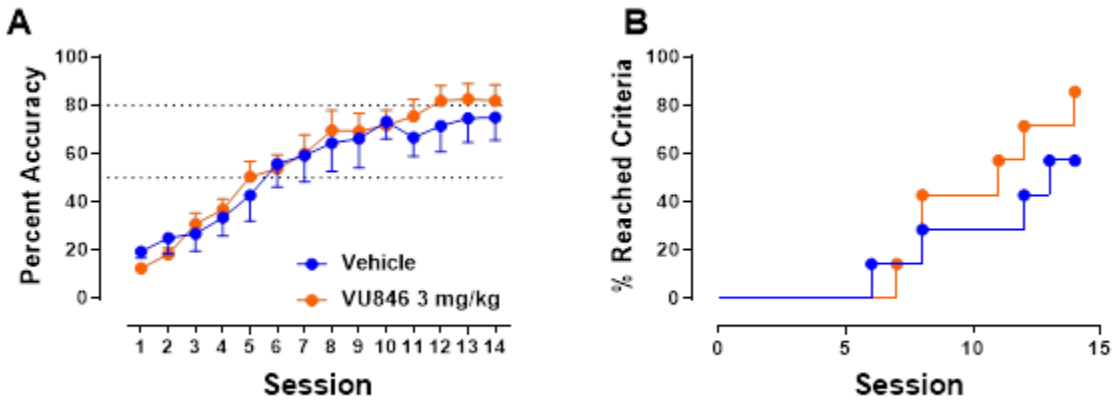

Figure A.2. VU0486846 3 mg/kg has no effect on acquisition of a reversal learning pairwise discrimination task in young mice. Shown is the percent accuracy over subsequent days following vehicle or VU0486846 (3 mg/kg) administration in young animals (A), and the percentage of animals who achieved 80% criteria for 2 consecutive days in the vehicle and VU0486846 (3 mg/kg) dosing. No significant differences between groups (mixed effects model). N=6-7/group

x time interaction (p=0.4686, $F_{(20,130)}$=0.9984) on acquisition. A main effect of time was observed (p<0.0001, $F_{(20,130)}$=11.51) (Figure A.3A). No effect of dose on percentage of animals reaching criteria at a given session was observed (p=0.1492, χ^2 = 2.080, df = 1) (Figure A.3B) on learning of a reported top-down pairwise discrimination paradigm.

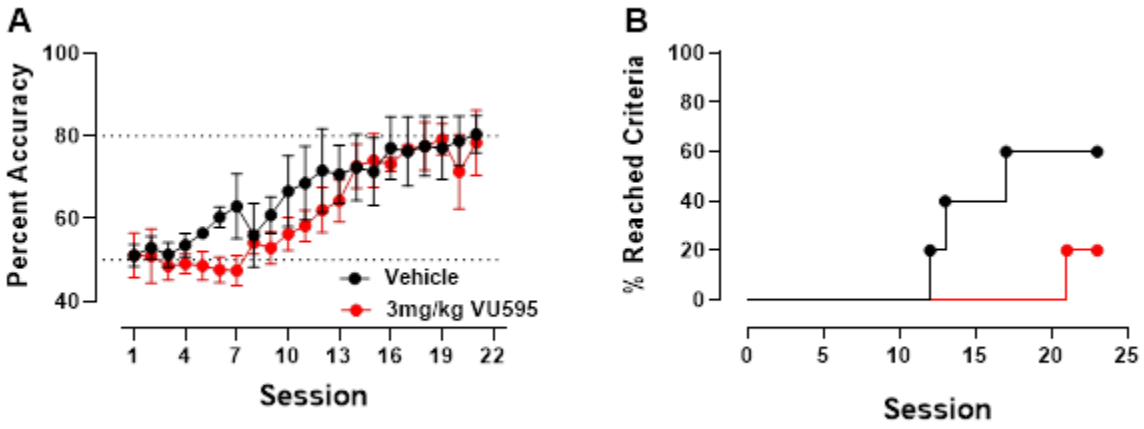

Figure A.3. VU0453595 3 mg/kg has no effect on acquisition of a pairwise discrimination task in young mice. Shown is the percent accuracy over subsequent days following vehicle or VU0453595 (3 mg/kg) administration in aged animals (A), and the percentage of animals who achieved 80% criteria for 2 consecutive days in the vehicle and VU0453595 (3 mg/kg) dosing. No significant differences between groups (mixed effects model). N=5/group

Discussion

VU0486846 dosed acutely at 3 mg/kg in young animals during the inactive period was demonstrated to enhance discrimination index in NOR. However, the assay proved to be variable and although visual trends towards cognitive enhancement was seen with donepezil this did not reach significant. The failure of VU0486846 to enhance NOR during the active phase suggests that there is a circadian difference in VU0486846 activity when dosed to young mice. This may be due to the well described inverted U of cholinergic activation (Dumas and Newhouse, 2011), where young mice will display reduced cholinergic activity during inactive phase which allows for pharmacological enhancement, but increased, optimal activity in the active phase which cannot be enhanced.

In the pairwise discrimination task neither VU0486846 in young animals nor VU0453595 in aged animals displayed enhancement of performance. This task has previously been demonstrated to be sensitive to M_1 muscarinic potentiation (Gould et al., 2015). It is important to note that in this publication the M_1 ago-PAM BQCA was used, whereas in young animals VU0486846, a PAM with no agonist activity was utilized (Rook et al., 2018). Potentially, agonist activity is required to demonstrate efficacy in the pairwise discrimination task. Additionally, both studies were a single dose study based on previously described efficacious doses with VU0486846 (Rook et al., 2018), and VU0453595 (Ghoshal et al., 2016), and studies in the NOR paradigm. However, these may not have been an appropriate dose for repeated dosing in the pairwise discrimination task. Further studies testing both higher and lower doses would be required. Additionally, pharmacokinetic studies following chronic dosing would indicate whether appropriate exposures relative to compound potency were obtained.

Appendix B: Measuring Central Cholinergic Structure with the PET Ligand [18F]-fluoroethoxybenzovesamicol in Preclinical Species

Introduction

Understanding central cholinergic integrity in clinical populations will be fundamentally important when considering M_1 or M_4 mAChR PAMs as treatments for the treatment of MCI or AD. In previous chapters I have discussed utilizing ChAT immunohistochemistry to measure central cholinergic structure in mice. This however is not a viable option in clinical trials, as such a more translational approach is desirable. Numerous radioisotopes have been developed targeting different cholinergic neuronal markers including AChE (Kikuchi et al., 2013) and the vesicular acetycholine transporter (vAChT) (Giboureau et al., 2010). Of these [18F]-fluroethoxybenzovesamicol ([18F]-FEOBV) displays ideal kinetic and binding properties for assessment in rodent, non-human primate and clinical studies (Aghourian et al., 2017; Mulholland et al., 1993, 1998; M. Parent et al., 2012; M. J. Parent et al., 2013). In the current section I will discuss attempts to validate [18F]-FEOBV for measuring central cholinergic structure in mice.

Methods

[18F]-FEOBV PET imaging

Young C57/b6 mice (3-month-old, male, Jackson Laboratories, n=6) and young Sprague Dawley rats (8-10-week-old, male, Taconic n=10) were implanted with jugular catheters with vascular access buttons. For mice an MRI compatible button was used. All animals were given 5-7 days to recover post operatively prior to imaging. For imaging, animals were anesthetized with 3% isoflurane and dosed with 18-23MBq (rats) or 11MBq (mice) of [18-F]-FEOBV via intravenous bolus. A 60-minute dynamic PET scan (Bioscan NanoSPECT/CT) was used to capture [18-F]-FEOBV uptake and clearance. A subset of animals (n=6, mice and n=3, rats)

received cold FEOBV (0.01 mg/kg) 30-minutes prior to scanning to block specific [18F]-FEOBV binding.

For analysis, in both mice and rats kinetic modeling was performed utilizing the cerebellar region as a reference region. In rats, regional activity was calculated using volumetric ROIs from a single rat anatomical template. In the mice, anatomical T2 MRIs were captured, and individualized volumetric ROIs were generated. Uptake was then expressed as % injected dose/volume of ROI (%ID/gram) and regional activity plotted on a time activity curve.

[18F]-FEOBV autoradiography

3-4-month-old C57/b6 mice were sacrificed brains were collected and immediately frozen on dry ice. 20um thick slices were cut on a cryostat and directly slide mounted with 2x PFC, 2x Striatum, and 2x Dorsal Hippocampus/thalamus sections on each slide. Slides were stored at -20C. On the day of the autoradiography study the slides were defrosted, and the sections were drawn around with a Pap pen so that each area for incubation had 1 of each brain area. Sections were incubated in 0.1M PBS solution for 20-minutes. On each slide, three sections underwent incubation with [18F]-FEOBV at 6.97MBq/L in PBS and three sections underwent incubation with [18F]-FEOBV at 6.97MBq/L and cold at 50x concentration for 20-minutes. Slides were washed by dipping three times in milliq water and dried. Imaging was performed by using radioactive counts over a 20-minute period (Biospace Lab Micro-Imager)

Results

[18F]-FEOBV PET imaging

Following IV administration with [18F]-FEOBV both mice (Figure B.1) and rats (Figure B.3) displayed a rapid uptake of [18F]-FEOBV into the brain with high levels of uptake in areas with rich cholinergic innervation (e.g., striatum).

Mice displayed relatively higher levels of uptake in the nucleus accumbans, thalamus and whole cortex, compared to the hippocampus and cortex (Figure B.2A). An overall effect of brain area

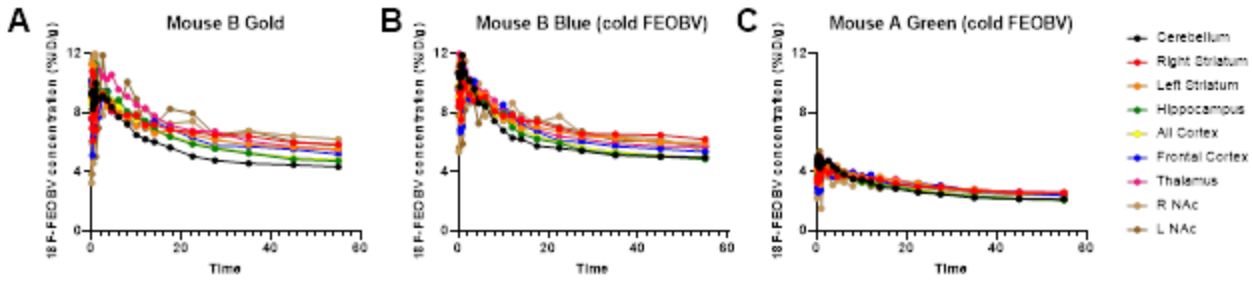

Figure B.1. Example time activity curves in mice following [18F]-FEOBV administration. Shown are 3 different mice, in (A) the mouse received just [18F]-FEOBV. In (B) and (C) the mice received [18F]-FEOBV and cold FEOBV 30-minutes prior to the dose of [18F]-FEOBV.

was observed ($p<0.0001$, $F_{(5,20)}=10.75$) However binding in these areas was not consistently blocked by cold FEOBV, with a significant reduction in binding only seen in the nucleus accumbans and the magnitude of binding reduction was small (Figure B.2B). An main effect of region and cold compound was observed ($p<0.0001$, $F_{(5,45)}=24.83$ and $p=0.0135$, $F_{(1,9)}=9.391$ respectively), no region x cold compound interaction was observed ($p=0.2752$, $F_{(5, 45)}=1.314$).

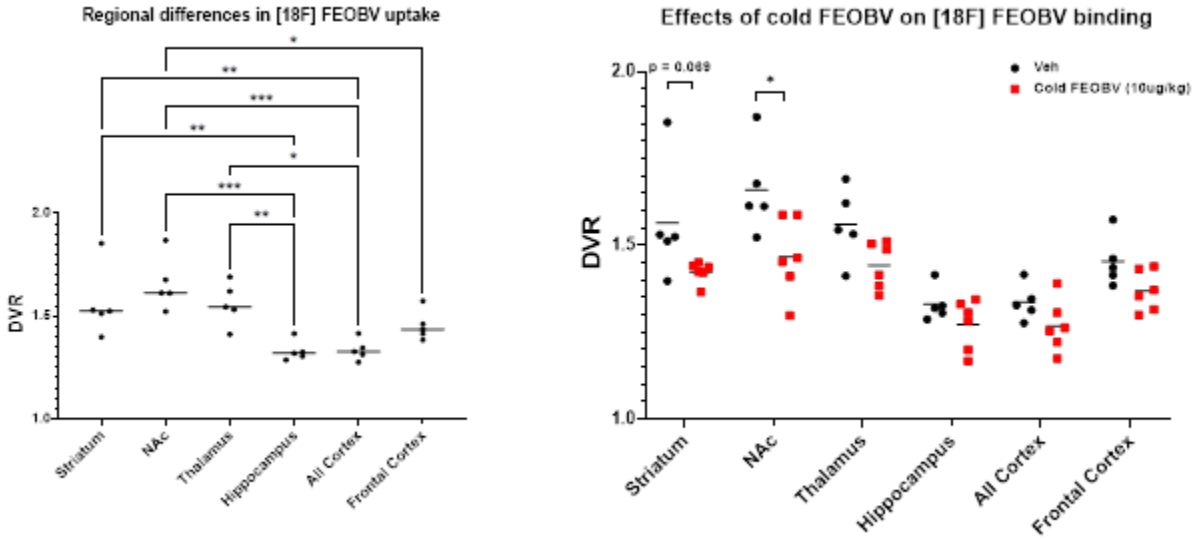

Figure B.2. Regional uptake of [18F]-FEOBV in mice. Shown in (A), and the ability of cold FEOBV to block specific [18F]-FEOBV binding in mice is shown in (B). * p<0.05, ** p<0.01

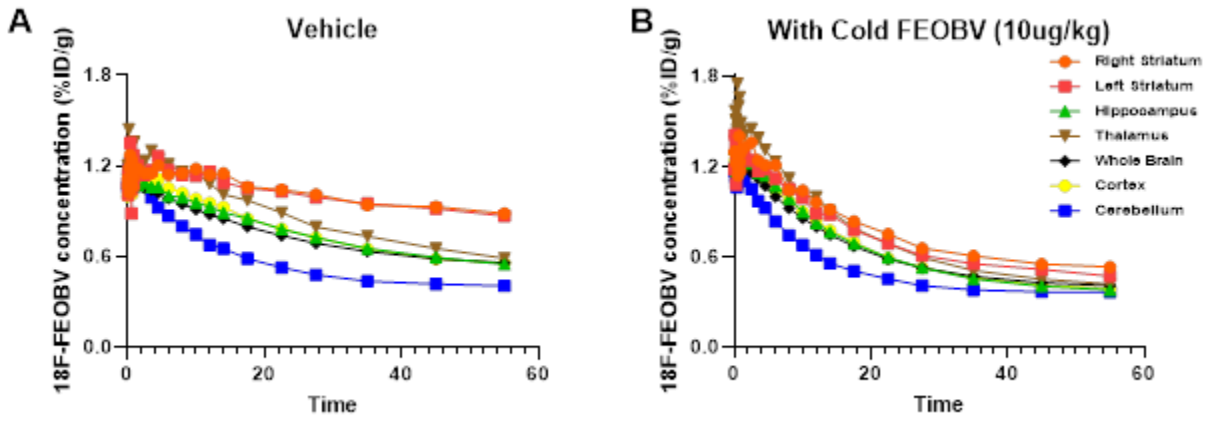

Figure B.3. Example time activity curves in rats following [18F]-FEOBV administration. Shown are 2 different mice, in (A) the rat received just [18F]-FEOBV. In (B) the rat received [18F]-FEOBV and cold FEOBV 30-minutes prior to the dose of [18F]-FEOBV.

The studies in rats revealed a larger relative difference in binding between the brain areas (Figure 4A), and large reductions in binding in the presence of the cold ligand (Figure B.4B) similar to published data (M. Parent et al., 2012). Due to the relatively low number of animals in the rat studies statistical analysis was not performed.

[18F]-FEOBV autoradiography

Following autoradiography binding studies [18F]-FEOBV regional binding did not demonstrate the expected pattern of binding described in the literature with areas of highest cholinergic innervation not revealing the highest [18F]-FEOBV binding, e.g., striatum (Figure B.5A). Concurrent incubation with saturating concentrations of the cold FEOBV ligand was able to reduce the binding of [18F]-FEOBV (Figure B.5B).

Discussion

[18F]-FEOBV PET imaging studies in rats replicated published data in the literature (M. Parent et al., 2012) (although in a smaller number of animals). Efforts to translate this into mice

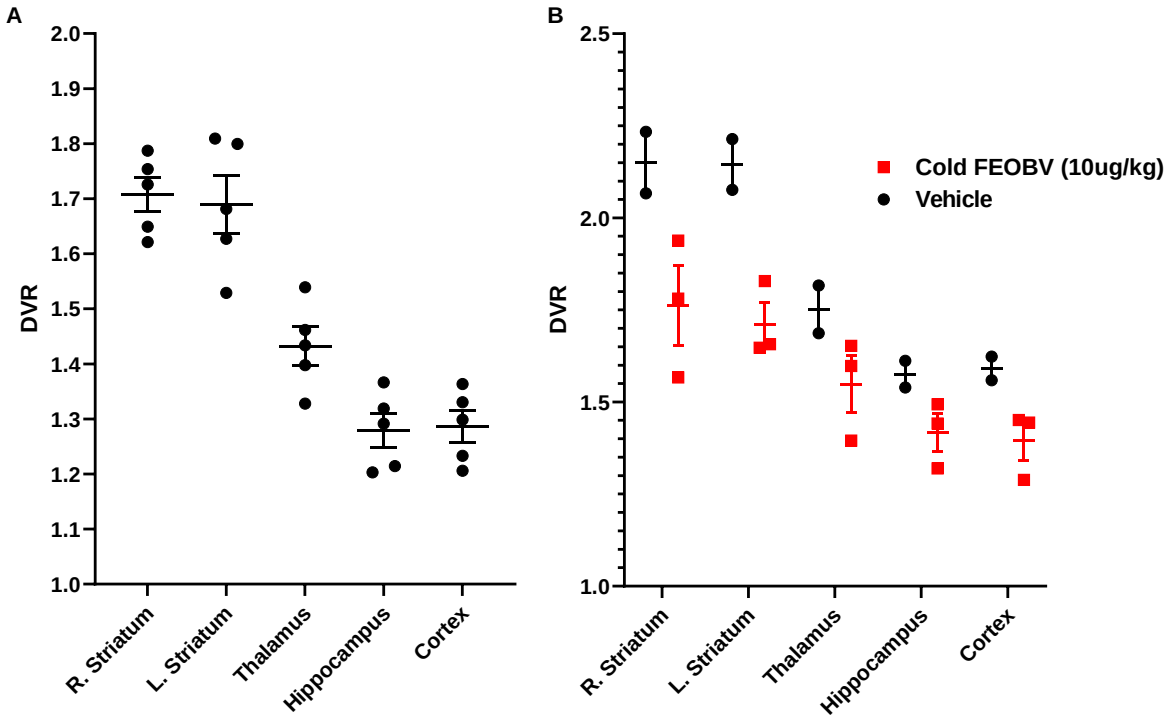

Figure B.4. Regional uptake of [18F]-FEOBV in rats. Shown in (A), and the ability of cold FEOBV to block specific [18F]-FEOBV binding in rats is shown in (B).

to enable comparisons of young to aged mice proved unsuccessful. Uptake between cholinergic-rich areas and areas with less cholinergic innervation broadly followed a similar pattern as was seen in rats, with higher uptake in striatal and thalamic areas, and reduced uptake in cortical and hippocampal areas. However, the magnitude of difference between these areas was smaller than observed in the rats. Importantly, cold FEOBV was not able to significantly reduce binding in most of the areas assessed. One reason for this may be due to an inability to increase the FEOBV dose to sufficiently saturating levels. As a vesamicol derivative FEOBV is a vAChT antagonist, where higher doses result in respiratory arrest.

Autoradiography was utilized to test whether higher concentrations of cold FEOBV would block [18F]-FEOBV-specific binding. These studies successfully demonstrated that saturating concentrations of FEOBV were able to block [18F]-FEOBV specific binding, however the binding pattern was not consistent with previous studies (Mulholland et al., 1998), that demonstrated high

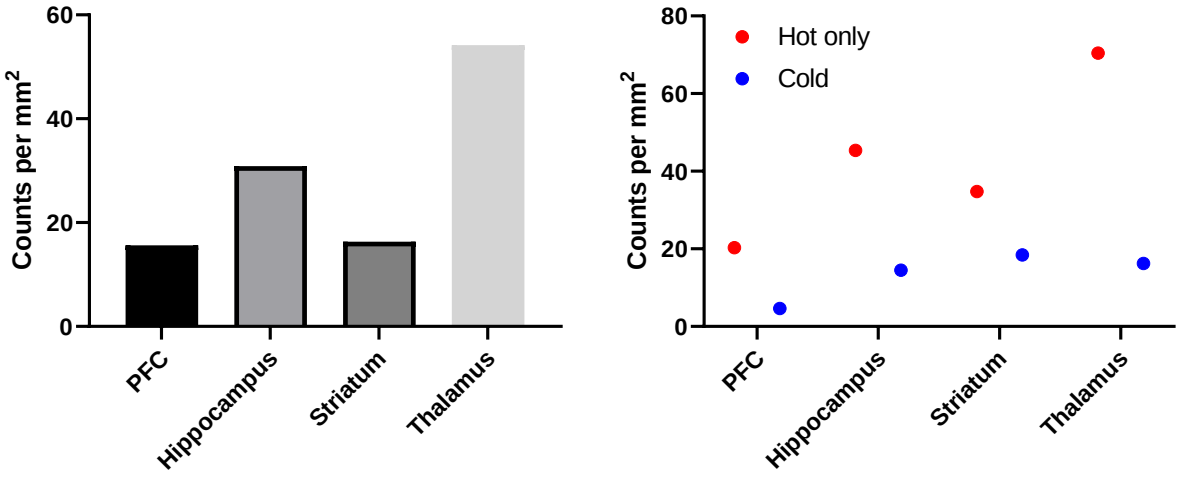

Figure B.5. Regional binding of [18F]-FEOBV in *ex vivo* mouse brain slices. Shown in (A), and the ability of cold FEOBV to block specific [18F]-FEOBV binding in *ex vivo* brain slices is shown in (B).

levels of binding in cholinergic-rich regions such as the striatum. The failure to replicate the high levels of binding in striatal areas seen in previous studies and the in vivo PET studies suggest a methodological problem either with tissue preparation or radiotracer incubation with the tissue.

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