Adenosinergic Modulation of Sleep-like Brain States Under Urethane Anesthesia

by

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Abstract

The reason and functional relevance of why we sleep continues to perplex researchers. One fundamental reason behind why sleep remains a mystery is because it is challenging to study given how easily it is disrupted. Anesthesia has been suggested to be a model for studying sleep given the behavioural and neurobiological similarities between the two states of unconsciousness. Sleep is often thought of as a unitary process, but it is highly dynamic. There are two distinct stages of sleep – NREM and REM. Cycling between these two states occurs in a periodic and rhythmic fashion over the course of our sleep episode. Unlike other anesthetics which promote a coma-like or at best a NREM-like unitary state, the acute laboratory anesthetic urethane closely imitates the forebrain activity dynamics of natural sleep. Under urethane, spontaneous cycling between a deactivated (NREM-like) and activated (REM-like) EEG pattern occurs, and these two states have peripheral physiological correlates that also mimic natural sleep. This makes urethane anesthesia a powerful model to study sleep-state dynamics which allows for the facilitated examination of brain mechanisms involved in sleep.

A variety of molecules have been suggested to act as “sleep factors” and contribute to both the onset and maintenance of sleep. Among these factors is adenosine, a highly potent sleep-promoting molecule that may be imperative for expression of sleep need. Adenosine exerts its influence on sleep via two receptors – the A1 and the A2A receptor. In this thesis, I tested the influence of adenosine, its antagonist caffeine, and two receptor specific agonists for A1 and A2A receptors to evaluate if parallel effects to sleep occur in sleep and the urethane model. It is crucial

that we continue to validate existing models of natural sleep to strengthen them as avenues to study sleep.

Male Sprague Dawley rats were anesthetized with urethane to surgical plane after which bipolar electrodes were used to record LFPs from the cortex and hippocampus. After establishing a baseline consisting of stable, cyclic alternations between the deactivated and activated state for at least one hour, either adenosine or caffeine was administered peripherally (IV) or centrally (ICV). Any changes in brain state alternations were compared to our baseline recording. My results indicated that adenosine promoted the deactivated state whereas caffeine decreased the deactivated state. These drugs were also co-administered in an attempt to challenge the effect of adenosine with its antagonist caffeine. This led to an interesting biphasic response in which an increase in the deactivated state was observed initially followed by a decrease in this state. I then tested the effect of central administration of adenosine A1 agonist CPA and A2A agonist CGS 21680. CGS 21680 lead to an increase in the deactivated state similar to what was observed with adenosine. CPA also increased the deactivated state, but this effect was extremely robust, and the activated state was eliminated for an extended period of time with some return of cycling observed towards the end of my experiments.

Cumulatively, my results indicate that adenosine and its agonists promote the deactivated state but that activation of A1 vs. A2A receptors mediate very different responses. A1 activation promotes the deactivated state much more powerfully than A2A activation. Caffeine on the other hand, decreases the deactivated state. Importantly, these results are consistent with effects on sleep state in natural sleep, which further validates the urethane model.

Preface

This thesis is an original work by Aakanksha Singh. The research projects, of which this this is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Cellular and Network Dynamics of Neo- and Limbic-Cortical Brain Structures”, AUP092.

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List of Abbreviations

ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
BF	Basal Forebrain
Ca^{2+}	Calcium ion
cAMP	Cyclic Adenosine Monophosphate
CGS 21680	2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarboxamidoadenosine-hydrochloride
CNO	Clozapine N-Oxide
CNS	Central Nervous System
CPA	N^6-Cyclopentyladenosine
CTX	Cortex
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
EEG	Electroencephalogram
GABA	gamma-Aminobutyric acid
GIRK	G Protein-Activated Inwardly Rectifying Potassium Channel
GFAP	Glial Fibrillary Acidic Protein
GPCRs	G Protein-Coupled Receptors
HPC	Hippocampus
HRCT	Hypocretin
ICV	Intracerebroventricular
IV	Intravenous

K^+	Potassium ion
LDT	Laterodorsal Tegmentum
LFP	Local Field Potentials
mRNA	Messenger Ribonucleic Acid
NAc	Nucleus Accumbens
NBTI	Nitrobenzylthioinosine
NREM	Non-Rapid Eye Movement
NMDA	N-Methyl-D-aspartic acid
OT	Olfactory Tubercle
PAM	Positive Allosteric Modulator
PF-LHA	Perifornical-Lateral Hypothalamic Area
PPT	Pedunculopontine Nucleus
PV	Parvalbumin
REM	Rapid Eye Movement
SO	Slow Oscillation
SWA	Slow-Wave-Activity
SWS	Slow-Wave-Sleep
TMN	Tuberomammillary Nucleus
UDP	Uridine Diphosphate
UTP	Uridine Triphosphate
VLPO	Ventrolateral Preoptic Area
VTA	Ventral Tegmental Area

Introduction

Sleep is an essential and dynamic process that is highly conserved across most animals. It is fundamental in order to maintain good health since loss of the quality or quantity of sleep can have deleterious physiological and psychological effects. In humans, it is estimated that approximately one third of our lives are spent sleeping. Although sleep takes up a substantial proportion of our existence, the ecological reason behind why we sleep remains contested and unclear. What is more clear, however, are the mechanisms by which sleep is produced.

The two-process model proposed by Borbély suggests that sleep is regulated via homeostatic and circadian mechanisms (Borbély & Neuhaus, 1979). Homeostatic regulation refers to the increase in sleep pressure occurring with increasing time spent awake while the circadian process refers to a biological rhythm that is entrained by light from our environment. The circadian rhythm is regulated by a structure known as the suprachiasmatic nucleus which is housed in the hypothalamus (Luppi & Fort, 2019). Light-sensitive neurons in our retina allow our internal biological clock to be synced with the day/night cycle and allow for this entrainment (Luppi & Fort, 2019). Homeostatic pressure, on the other hand, is thought to be regulated by other structures in the hypothalamus such as the GABAergic VLPO which is a powerful, sleep-promoting nuclei, and the histaminergic TMN which is a crucial arousal center (Luppi & Fort, 2019).

Part of the reason why sleep remains mysterious is because it is easily disrupted. This makes it challenging to study. Interestingly, nuclei implicated in sleep-wake regulation (such as the VLPO and TMN mentioned above) have also reported to be

neuroanatomical targets of anesthetic agents (Adapa, 2017; Franks, 2008; Ward-Flanagan & Dickson, 2019). This suggests that anesthesia can be a powerful model to study sleep which allows for more controlled experimentation (Clement et al., 2008; Mashour & Pal, 2012; Tung & Mendelson, 2004; Ward-Flanagan & Dickson, 2019).

In addition to the involvement of particular sleep-wake nuclei, it has been reported that certain *compounds* can also promote sleep. One well known example of this is the hormone melatonin which is released from the pineal gland and is linked to the circadian cycle (Cajochen, Chellappa, & Schmidt, 2010). Moreover, purines are another class of compounds that have been proposed to play a role in sleep.

Purines

Purines are a class of ubiquitous molecules and organic compounds found in our central and peripheral nervous system that have integral roles in metabolism and signal transduction (Hu et al., 2013) in addition to regulating the immune response (Cekic & Linden, 2016). Fusion of an imidazole ring to a pyrimidine ring makes up the general structure of these heterocyclic, aromatic compounds with nitrogenous bases (Ouellette & Rawn, 2018). Purinergic signalling has been present since the beginning of evolution and was functional even in single cell organisms (Verkhratsky & Burnstock, 2014). When cells are damaged, there is an increase in the release of purines which act as a "damage signaller" (Verkhratsky & Burnstock, 2014). Therefore, it has been suggested that purinergic receptors on cell surfaces initially evolved when extracellular concentrations of purines increased due to cell damage as a mechanism for cytoprotection thus giving purines a target for binding (Cekic & Linden, 2016).

There are several classes of purinergic receptors including P1, P2X, and P2Y which are all G protein-coupled receptors (GPCRs) (Cekic & Linden, 2016). P1 receptors, also known as adenosine receptors, are metabotropic and adenosine activated (Cekic & Linden, 2016). P2Y receptors are also metabotropic and have a variety of ligands including ATP, ADP, UTP, UDP, and UDP-glucose (Cekic & Linden, 2016). In contrast, P2X receptors are ionotropic and activated by ATP (Cekic & Linden, 2016). Within P1 receptors, there are four classes of adenosine receptors including A1, A2A, A2B, and A3 receptors which are encoded by the ADORA1, ADORA2A, ADORA2B, and ADORA3 genes, respectfully (Cekic & Linden, 2016). A1 and A3 receptors are G_i/G_o coupled, whereas A2A and A2B receptors are G_s coupled (Cekic & Linden, 2016). For the purposes of this review, we will be focused on adenosine receptors specifically.

It is essential to note that adenosine is not defined as a classic neurotransmitter. It is not stored in or released from synaptic vesicles and it is not released via exocytosis (Fredholm, Chen, Cunha, Svenningsson, & Vaugeois, 2005). Additionally, its site of action is not restricted to the synapse (Fredholm et al., 2005). Instead, it is best described as a neuromodulator and a homeostatic messenger and has the ability to influence both neurotransmitter release and neuronal excitability (Fredholm et al., 2005).

Sources and Regulation of Adenosine

There are a variety of mechanisms by which adenosine can arise in the extracellular space in the brain. Adenosine levels are influenced by both glial and neuronal activity (Bjorness & Greene, 2009; Pascual et al., 2005). Largely speaking, adenosine can be released from cells via transporters after an increase in intracellular concentrations and also by the extracellular conversion/dephosphorylation of adenine

nucleotides (mainly ATP) by ecto-nucleotidases (Chen, Lee, & Chern, 2014; Dunwiddie & Masino, 2001; Fredholm et al., 2005; Huang, Urade, & Hayaishi, 2011). In the CNS, the clearance of adenosine is dependent primarily on two enzymes: adenosine kinase and adenosine deaminase (Fredholm et al., 2005; Huang et al., 2011). Extracellularly, adenosine is cleared via equilibrative nucleoside transporters or by the breakdown of adenosine into inosine by adenosine deaminase. Moreover, extracellular ATP can be converted to AMP by a specific ecto-5'-nucleotidase known as CD39 after which AMP can be converted to adenosine via CD73 (another ecto-nucleotidase). The adenosine can then be converted to inosine by adenosine deaminase as mentioned earlier (Chen et al., 2014; Fredholm et al., 2005; Huang et al., 2011). It has been reported that neurons express high concentrations of adenosine kinase while astrocytes are enriched in adenosine deaminase (Fredholm et al., 2005).

Intracellularly, ATP can be transformed to adenosine which involves the conversion of ATP to ADP via ATPases and then to 5'-AMP by adenylate kinase (Dunwiddie & Masino, 2001; Fredholm et al., 2005). Adenosine can then be formed from 5'-AMP via cytosolic 5'-nucleotidase after which adenosine kinase can convert it back 5-AMP (Dunwiddie & Masino, 2001; Fredholm et al., 2005). An additional pathway for the intracellular metabolism of adenosine involves S-adenosylhomocysteine but this has been reported to be significant only in cardiomyocytes (Fredholm et al., 2005). **Figure 1** illustrates a simplified version of the source and regulation of adenosine.

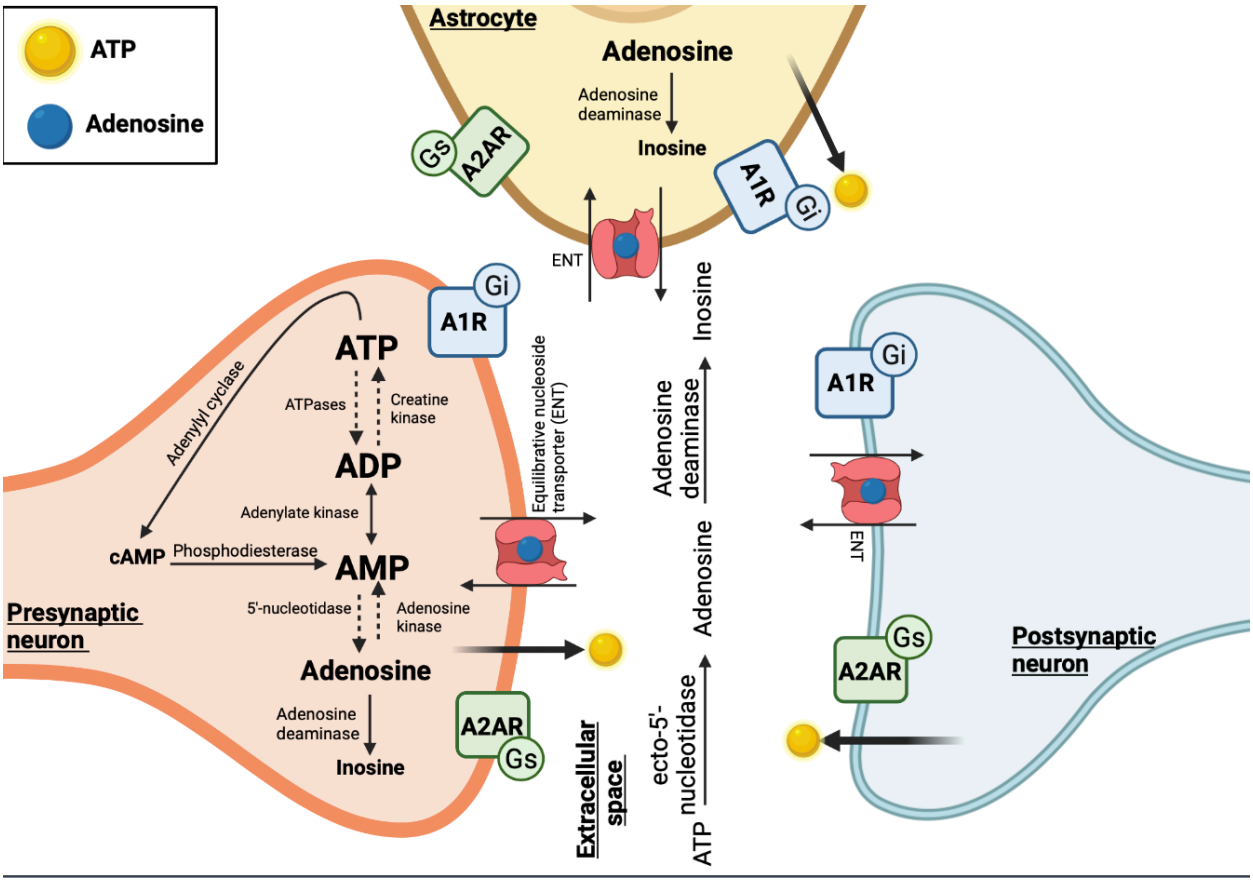

Figure 1. Schematic representation of the sources and regulation of adenosine

Adenosine involvement in biological systems

As an omnipresent compound in the body, adenosine has a variety of functions. In conjunction to its involvement in metabolism, it is frequently used in the clinical practice of anesthesiology and critical care medicine as a constituent of the presurgical and postsurgical timeline (Eltzschig, 2009). In medicine, adenosine is an antiarrhythmic drug used as a form of treatment for supraventricular tachycardia (Eltzschig, 2009). Additionally, it serves as a platelet aggregation inhibitor and an arterial vasodilator (Eltzschig, 2009). Moreover, in the ischemic heart, all four adenosine receptors have been implicated in ischemic preconditioning and A2A receptor activation has been suggested to reduce the locus of myocardial infarction (Cekic & Linden, 2016). In

patients, adenosine administration prior to stenting revealed a reduction in myocardial infarct size (Micari et al., 2005).

Studies have indicated that adenosine behaves as a distress signal and that extracellular expression of adenosine receptors increases in the presence of cellular injury or hypoxic conditions (Eltzschig, 2009). Of the four adenosine receptors, A2A and A2B receptors specifically are upregulated in response to immune cell activation and serve to minimize inflammation post tissue injury (Cekic & Linden, 2016). Unlike A2A receptors which are expressed in most immune cells, A2B receptors are predominantly expressed in macrophages and dendritic cells (Cekic & Linden, 2016). Although A2A receptors have a higher affinity for adenosine, A2B signalling becomes pertinent in inflamed tissues under hypoxic conditions and contributes to the chronic phase of recovery following injury (Cekic & Linden, 2016). This is because A2B receptor facilitated healing stimulates the synthesis of mediators such as interleukin-6 and vascular endothelial growth factor more vigorously than A2A receptor activation (Cekic & Linden, 2016). Thus, adenosine signalling is an integral and protective component of recovery, especially under hypoxic conditions. In fact, in both rodents and humans, extracellular adenosine is significantly elevated in the presence of hypoxia (Eltzschig, 2009). Furthermore, hypoxia-inducible factor (HIF) is paramount in facilitating hypoxia-provoked adenosinergic responses and hypoxia-induced changes in extracellular adenosine signalling events include an upregulation in adenosine production and a concomitant transcriptional downregulation of adenosine transporter (Eltzschig, 2009). Therefore, an overall increase in extracellular adenosine signalling highlights the

significance of hypoxia-elicited changes in adenosine which mitigates inflammation, attenuates tissue-damage, and discourages organ-dysfunction.

Arguably, one of the most intriguing roles of adenosine is its involvement in sleep. Humans spend about one-third of their lives sleeping and sleep is unequivocally essential for good mental, emotional, and physical health. Sleep disturbances are often comorbid with other illnesses and a decline in the quality and quantity of sleep has been linked to neurodegeneration, cognitive deficits, impaired decision making, and decreased executive function (Elmenhorst et al., 2017). Adenosine, when administered exogenously, promotes the initiation and maintenance of sleep (Basheer, Strecker, Thakkar, & McCarley, 2004; McCarley, 2007). Indeed, the extracellular concentration of adenosine increases as a function of time spent awake, suggesting that it plays an essential role as a physiological trigger for sleep. Furthermore, and perhaps the most well-known argument for an adenosinergic involvement in sleep comes from the common and world-wide use of caffeine (an antagonist of adenosine) to promote arousal and wakefulness and combat sleepiness.

Adenosine and its involvement in sleep promotion

The somnogenic effect of adenosine was first reported in 1954 in cats and it has since been widely characterized as a sleep substance (Feldberg & Sherwood, 1954). To this day, there is growing evidence that adenosine plays a paramount role in the initiation and maintenance of sleep, making it a valuable tool to examine sleep-wake architecture (Basheer et al., 2004; McCarley, 2007; Yuan et al., 2017). Because extracellular concentrations of adenosine are influenced by neuronal activity, there is an increase in adenosine levels throughout the day as a result of the metabolic breakdown of ATP and

cAMP (Hu et al., 2013). As a neuromodulator that naturally increases in the brain with prolonged wakefulness, adenosine is hypothesized to be an essential regulator in the homeostatic drive for sleep (Basheer et al., 2004; McCarley, 2007; M.M. Thakkar, Engemann, Walsh, & Sahota, 2008). Furthermore, adenosine has been suggested to promote non-rapid eye movement (NREM) sleep specifically, which is characterized by high-amplitude, low-frequency electrophysiological activity known as slow-waves (Korkutata et al., 2019; Oishi, Huang, Fredholm, Urade, & Hayaishi, 2008; Oishi et al., 2017). The intensity of slow wave activity (SWA), which is characterized to be within the frequency range of 0.5-4.5 Hz, is strongly correlated with the previous duration of waking and extracellular adenosine concentrations (Bjorness et al., 2016; Greene, Bjorness, & Suzuki, 2017; Lazarus, Oishi, Bjorness, & Greene, 2019). SWA is expressed during NREM sleep and is considered a "marker for sleep homeostasis" (Greene et al., 2017) because it increases with high sleep need, and decreases with low sleep need (Lazarus et al., 2019). In order to understand the influence of adenosine on sleep, it is crucial to examine what is happening at the level of its receptor and it has been suggested that both the A1 and A2A receptors are of particular significance in delineating this somnogenic effect. These receptors fall under the category of P1 receptors which are metabotropic, G-protein-coupled and adenosine-activated (Cekic & Linden, 2016).

Dissociable effects of A1 vs. A2A receptors

Adenosine acts as a neuromodulator through G-protein coupled receptors that acts as a "gating mechanism" (Lazarus et al., 2019) of SWS-SWA expression, and thus sleep homeostasis, by regulating arousal. Its involvement on sleep-wake circuitry is primarily mediated via A1 and A2A receptors (Lazarus et al., 2019; Van Dort, Baghdoyan,

& Lydic, 2009). Of the four adenosine receptors, the A1 and A2A receptors are most widespread in the central nervous system (CNS) with the A2A receptor having a more restricted distribution (Svenningsson, Nomikos, Ongini, & Fredholm, 1997). These receptors have been classified based on their ability to either inhibit (A1), or stimulate (A2) adenylate cyclase (de Lera Ruiz, Lim, & Zheng, 2014; Fredholm et al., 2005). A2A receptors are most densely localized in the nucleus accumbens, olfactory tubercle, globus pallidus, and caudate putamen (Svenningsson et al., 1997). In contrast, A1 receptors are widely distributed in almost all brain areas with the hippocampus, striatum, cortex, and thalamic nuclei having the highest levels of expression (Elmenhorst et al., 2017; Svenningsson et al., 1997).

In a model characterized by Lazarus et al. (2019), A1 receptors regulate sleep maintenance and are imperative for expression of sleep-need while A2A receptors provide sleep-gating. Given the differential role of these receptor subtypes, it is essential to dissociate the arousal state from sleep homeostasis in order to better understand the action of adenosine. Adenosine interaction with A1 receptors increases sleep and SWA (Radulovacki, Virus, Djuricic-Nedelson, & Green, 1984). A1 receptors also mediate sleep homeostasis based on “astrocytic gliotransmission” which suggests the involvement of a glial-neuronal circuit (Bjorness et al., 2016). An increase in extracellular adenosine in response to increased metabolic demand also promotes an increase in synaptically released glutamate which activates NMDA receptors (Brambilla, Chapman, & Greene, 2005; Greene et al., 2017). This NMDA receptor activation increases inward currents and influx of Ca^{2+} which in turn increases extracellular adenosine thus increasing interaction with A1 receptors (Brambilla et al., 2005; Greene et al., 2017). This increase in adenosine

then acts presynaptically to reduce presynaptic glutamate release via a negative feedback loop (Bjorness et al., 2016; Brambilla et al., 2005). Postsynaptically, adenosine increases inwardly rectifying potassium conductances and reduces I_h conductance (Bjorness et al., 2016; Rainnie, Grunze, McCarley, & Greene, 1994). When adenosine activates A1 receptors, there is an increase in SWA due to "[increased] G-protein inward rectifying current, decreased hyperpolarization-activated inward current, and increased presynaptic inhibition" (Greene et al., 2017). It has been reported that A1 receptor activation modulates SWA by two distinct mechanisms. These mechanism are referred to as "direct" and "indirect" mechanisms (Lazarus et al., 2019). The direct mechanism of adenosinergic SWA control is mediated via the presynaptic inhibition of both thalamic and cortical neurons which leads to "functional deafferentation" and subsequently enhances slow oscillations (SO) in thalamocortical neurons (Lazarus et al., 2019). This increase in SO is mediated by A1 receptor induced decrease in hyperpolarization activated currents and increased in GIRK channel conductance (Lazarus et al., 2019). In contrast, the indirect mechanism refers to A1 receptor facilitated inhibition of cholinergic neurons leading to a reduction in cholinergic tone and subsequently, arousal (Lazarus et al., 2019). The additive influence of these two mechanisms encourages the expression of SWA.

Evidence suggests that A2A receptors promote sleep by a different mechanism – by inhibiting the of activity of arousal centers in the brain (Lazarus et al., 2019). Unlike A1 receptors which have been argued to be responsible for sleep homeostasis, activation of A2A receptors has been argued to be essential for the induction of sleep. For example, infusion of the selective A2A receptor agonist CGS 21680 into the basal

forebrain of rats promotes sleep by concomitantly increasing GABA release in the TMN and thus reducing histamine release in the frontal cortex via inhibition of this prominent histaminergic system (Hong et al., 2005).

Taken together, adenosinergic regulation of sleep continues to be nuanced and it is critical to recognize and appreciate both the homeostatic and arousal components in order to better understand its effect on sleep promotion globally.

A1 Receptor Studies

A study investigating the effect of a selective A1 receptor agonist N^6-Cyclopentyladenosine (CPA) revealed a dose-dependent increase in EEG delta power (SWA) in NREM sleep in rats (Benington, Kodali, & Heller, 1995). Importantly, this effect was similar to what was seen when these rats were sleep deprived. Similarly, a 1996 study showed that CPA administration in rats increased SWA in NREM sleep (Schwierin, Borbély, & Tobler, 1996). Moreover, infusion of NBTI, an adenosine equilibrative transport blocker, decreased the activity of wake-active basal forebrain neurons (Alam, Szymusiak, Gong, King, & McGinty, 1999). This would make sense since an adenosine equilibrative transport blocker would increase the concentration of extracellular adenosine thus increasing its interaction with its receptors. In contrast, infusion of an A1 receptor antagonist increased the discharge rate of these neurons (Alam et al., 1999).

Furthermore, a 2003 study by Thakkar et al. revealed that the A1 receptor is a key component in adenosine exerting its homeostatic control of sleep by implementing the use of antisense technology. This technology has certain advantages which include localized and reversible knock-downs in conjunction with high receptor subtype

specificity (Thakkar, Winston, & McCarley, 2003). Perfusion of antisense oligonucleotides into the basal forebrain of freely behaving rats against A1 receptor mRNA was executed to prevent the translation of mRNA into receptor proteins (Mahesh M. Thakkar et al., 2003). The observed outcome of this was an overall reduction in NREM sleep and an increase in wakefulness (Thakkar et al., 2003). These researchers then sleep deprived the animals and the rats that had been treated with the antisense oligonucleotide spent significantly less time in NREM sleep compared to controls (Thakkar et al., 2003). Importantly, there was an increase in wakefulness with a simultaneous marked decrease in delta power (60-75%) (Thakkar et al., 2003). These findings suggest the involvement of the A1 receptor in promoting a NREM-sleep state.

Brambilla and colleagues showed cholinergic centres such as the LDT, PPT, and BF are under tonic inhibitory control of adenosine via A1 receptors (Brambilla et al., 2005). Their work indicated the presence of a negative feedback loop in which LDT glutamate release increased extracellular adenosine which then acted presynaptically to decrease glutamate and excitatory neurotransmitter release via interaction with the A1 receptor (Brambilla et al., 2005). Electrophysiology studies *in vitro* also revealed that A1 receptor activation with adenosine had an inhibitory postsynaptic effect on neurons in the LDT and basal forebrain (Arrigoni, Chamberlin, Saper, & McCarley, 2006; Rainnie et al., 1994).

Fascinatingly, a study conducted in humans revealed a decrease in A1 receptor availability after 14 hours of sleep recovery following 52-hour sleep deprivation (Elmenhorst et al., 2017). Although this decrease in A1 receptor was found in multiple

brain regions, the most dominate decrease was seen in the thalamus and striatum (Elmenhorst et al., 2017).

Many studies investigating the influence of adenosine on sleep have focused on the basal forebrain but it is suggested that there may be other regions of interest. One such example comes from examining the role of A1 receptors on hypocretin (HCRT) neurons in the perifornical-lateral hypothalamus area (PF-LHA) which are active during waking and quiescent during sleep (Alam et al., 2009). In this study, researchers bilaterally perfused both an adenosine agonist (CPA) and antagonist (CPDX) into the PF-LHA of rats. As predicted, administration of the antagonist CPDX increased overall waking and decreased both NREM and REM sleep (Alam et al., 2009). In contrast, application of the agonist CPA resulted in a decrease in overall wakefulness and an increase in REM and NREM sleep – with a particular effect seen on NREM sleep (Alam et al., 2009). Therefore, adenosinergic control of sleep could be facilitated, at least in part, via A1 receptors which inhibit HCRT neurons of the PF-LHA to promote sleep. The anatomy is of interest here because HCRT neurons project considerably to the basal forebrain which is an area implicated in arousal (Alam et al., 2009). A study conducted by Oishi and colleagues revealed that A1 receptors are coexpressed with adenosine deaminase in the TMN which is known to be an arousal centre (Oishi et al., 2008). Activation of these A1 receptors in the TMN increased NREM sleep thus decreasing arousal (Oishi et al., 2008).

Collectively, these studies indicate that not only is adenosine is key component in sleep management but that it is exerting a homeostatic effect via the A1 receptor with the basal forebrain being a key region of interest.

A2A Receptor Studies

Previous literature has reported that a subset of neurons in the core of the nucleus accumbens (NAc) are integral in mediating slow-wave-sleep (Oishi et al., 2017). This was illustrated by optogenetically and chemogenetically exciting the A2A receptor in the NAc core which led to a subsequent increase in SWS. These researchers wanted to further verify the role of the NAc core in sleep-wake architecture and implemented the use of inhibitory DREADDs to suppress neuronal activity in this region. Their results indicated a significant reduction in total SWS and in SWS episode number. Interestingly, when the same inhibitory DREADD was used to examine homeostatic sleep rebound after sleep deprivation, there was no significant difference observed in CNO-induced decrease of SWS in the sleep-deprived and non-sleep-deprived groups. This indicates that A2A receptors in the NAc core promote sleep independent of homeostatic sleep pressure (Oishi et al., 2017). This is significant because this further suggests that homeostatic sleep pressure may be regulated by A1 receptors.

In 2001, a study by Scammell et al. revealed that A2A receptor agonist CGS 21680 (a highly selective and potent A2A receptor agonist) increased NREM sleep and c-FOS expression in the VLPO while concomitantly decreasing c-FOS in the TMN (Scammell et al., 2001). They hypothesized that A2A receptors on the shell of the nucleus accumbens were likely activated by CGS 21680 which then increased the activity of sleep-promoting neurons in the VLPO (Scammell et al., 2001).

A 2019 study by Zhou et al. also provided evidence in support of the essential role of NAc core neurons in promoting SWS. They investigated the role of astrocytes in the ability of the NAc to mediate SWS by cytotoxic ablation of glial fibrillary acidic protein

(GFAP)-positive cells (likely astrocytes) in the NAc core by diphtheria toxin administration. In wild-type mice, there was an increase in SWS (for several days) and extracellular adenosine following ablation of GFAP-positive cells in the NAc core. In contrast, SWS increase was not reported in A2A knock-out mice (Zhou et al., 2019). Taken together, their data suggested that increased levels of adenosine acting on A2A receptors in the NAc core stimulated SWS (Zhou et al., 2019).

Moreover, the olfaction system has also been reported to have an influential effect on sleep as smell and sleep have an intertwined relationship (Li et al., 2020). In the aforementioned study, activation of the olfactory tubercle (OT) with DREADDs, photostimulation, and agonist administration showed that A2ARs in the OT promote NREM sleep in rats. In contrast, administration of an antagonist, KW6002, resulted in a decrease in NREM sleep. With the use of anterograde tracing methods, the authors were able to identify that OT A2AR neurons formed inhibitory innervations with GABAergic neurons in the ventral pallidum and lateral hypothalamus thus highlighting a potential pathway for how the OT may promote sleep via these A2ARs (Li et al., 2020).

One study examined the effect of administering CGS 21680 to various regions in the rat brain. Results of this study indicated that the most effective way to promote sleep using this agonist was to administer it to the rostral basal forebrain (Satoh et al., 1999). Administration of this agonist to this region significantly increased FOS expression in the shell of the nucleus accumbens and the olfactory tubercle (both of which are regions where A2A receptors are highly expressed) (Satoh et al., 1999).

Unlike numerous studies employing the use of agonists and antagonists, Korkutata et al. (2019) ingeniously synthesized and utilized a novel A2A receptor positive

allosteric modulator (denoted A2AR PAM-1) to investigate adenosine interaction with the A2A receptor. Advantageously, the actions of A2A PAM-1 were limited to both the timing and site of adenosine release allowing it to enhance the natural adenosinergic effect. Additionally, positive allosteric modulators differ from traditional agonists because they bind to the desired receptor and increase receptor efficacy and affinity for the natural ligand. Both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) administration of A2A PAM-1 resulted in an increase in SWS in a dose-dependent manner without affecting body temperature, blood pressure, or heart rate which is usually seen with application of agonists (Korkutata et al., 2019). To confirm that this effect was as a result of interaction with the A2A receptor, these researchers applied the use of both A2A antagonists and knockout mice. Their results revealed a significant increase in SWS post-administration in wildtype mice, but this effect was not seen in the presence of an A2A antagonist or in knockout mice (Korkutata et al., 2019). This indicates that the A2A receptor was crucial in promoting the observed sleep effect.

Further evidence of A2A receptor involvement in sleep induction comes from a 2005 study in which CGS21680 was administered anesthetized rats to elucidate the sleep-promoting mechanism of adenosine at this receptor (Hong et al., 2005). The effect of CGS21680 on sleep and histamine release from the tuberomammillary nucleus (TMN) was investigated because in contrast to adenosine, enhancement of histaminergic activity from the TMN promotes arousal (Hong et al., 2005). Results revealed that agonistic action of CGS21680 promoted sleep by increasing GABA release in the TMN via inhibition of histamine system, thus providing a potential mechanism for the somnogenic effect of adenosine. (Hong et al., 2005).

A more recent study, highlighted the contribution of a specific neuronal circuit comprised of striatal A2A neurons and inhibitory synapses formed by parvalbumin neurons (PV) innervating the external globus pallidus in sleep promotion (Yuan et al., 2017). Using a variety of techniques including chemogenetics, optogenetics, immunoelectron microscopy, EEG, and controlled lesions, these researchers were able to show that the activation of striatal A2A neurons induced NREM sleep whereas inhibiting led to a reduction in NREM sleep. Anatomical mapping revealed that these striatal A2A neurons formed inhibitory synapses with PV neurons of the external globus pallidus thus implicating yet another sleep-promoting pathway under adenosinergic influence (Yuan et al., 2017).

Taken together, these studies suggest different mechanisms and regions implicated in the adenosinergic modulation of sleep by both A1 and A2A receptors in the brain. As initially suggested by Lazarus et al. (2019), it is necessary to evaluate a unified model of sleep-modulation at both receptor subtypes in order to comprehensively understand the role of adenosine in sleep modulation. Therefore, it is imperative for future studies to bridge the gap between adenosine interaction at both these receptors to elucidate its sleep-promoting effect globally.

Sleep and Anesthesia

Past research has suggested the involvement of the same sleep-wake circuitry across both sleep and anesthesia (Adapa, 2017; Clement et al., 2008; Franks, 2008; Ward-Flanagan & Dickson, 2019). There are several characteristics of sleep that make it a good explanatory metaphor for anesthesia including unconsciousness, decreased sensory awareness, and immobility (Mashour & Pal, 2012). It has been reported that

anesthetic intervention can attenuate sleep debt following sleep deprivation while sleep deprivation can modulate anesthetic potency (Tung et al., 2002, Tung at al., 2004). Additionally, endogenous sleep pathways provide neuroanatomical targets for anesthetic agents to exert their actions (Franks, 2008; Leung, Luo, Ma, & Herrick, 2014; Lydic & Baghdoyan, 2005; Mashour & Pal, 2012; Nelson et al., 2002; Ward-Flanagan & Dickson, 2019). GABAergic, histaminergic, noradrenergic, and cholinergic mechanisms have all been implicated in providing a common interface for sleep and anesthesia across several regions including the hypothalamus and brain stem (Mashour & Pal, 2012; Ward-Flanagan & Dickson, 2019).

Rationale

The focus of this study was to evaluate the similarity of adenosinergic action in urethane anesthesia and natural sleep. Anesthesia is a powerful and controllable tool to examine the modulation of sleep-like states. This is imperative as natural sleep is endogenously regulated and can be easily disrupted making controlled experimentation difficult and potentially unethical. Previous work in our lab has shown that urethane anesthesia at a surgical plane produces the same electroencephalographic (EEG) alternations between activated and deactivated brain states which are highly similar to those seen in natural sleep (Clement et al., 2008; Pagliardini, Gosgnach, & Dickson, 2013; Silver, Ward-Flanagan, & Dickson, 2021; Ward-Flanagan & Dickson, 2019; Whitten, Martz, Guico, Gervais, & Dickson, 2009). This, along with other commonalities makes urethane anesthesia an ideal model for both sleep and sleep-state dynamics (Clement et al., 2008; Pagliardini, Greer, Funk, & Dickson, 2012a; Ward-Flanagan & Dickson, 2019; Whitten et al., 2009). This ability of urethane to mimic natural sleep

without compromising state alternations makes it a formidable anesthetic which can be used to investigate the influence of a variety of compounds on sleep state dynamics. Given the high degree of similarity in brain state alternations between urethane anesthesia and natural sleep, urethane is the ideal anesthetic to evaluate adenosinergic action.

General Aims

The general aim of this study was to determine if the effects of adenosinergic signaling on forebrain state are similar under urethane anesthesia and natural sleep. I investigated the role of adenosine, its antagonist, caffeine, and selective A1 and A2A receptor agonists using a tripartite approach.

1. Does adenosine potentiate an NREM-like state under urethane anesthesia?

Brain state alternations in rats under urethane anesthesia were monitored via EEG both before and after systemic and intracerebroventricular (ICV) adenosine administration. ICV administration ensured that any effects observed were a result of the interaction with adenosine receptors in the brain as opposed to any peripheral action. The aim here was to determine the effects of adenosine in promoting particular forebrain states and to verify whether adenosine administration potentiates the deactivated (NREM-like) state.

2. Evaluating the action of caffeine – a nonselective, adenosine receptor antagonist under urethane anesthesia

The effects of both systemic and ICV administration of the competitive and nonselective adenosine antagonist, caffeine, on brain state alternations was examined.

We hypothesize that application of caffeine will depress the deactivated state or perhaps affect the incidence and duration of transitional brain states thus affecting the cyclic alternations between the activated and deactivated state.

3. Evaluating the action of A1 and A2A receptor agonists under urethane anesthesia

Additional experiments were conducted to observe the effects of targeted administration of selective adenosine receptor agonists including N^6-cyclopentyl adenosine (CPA) (A1 agonist) and CGS 21680 (A2A agonist). Both agonists of adenosine were predicted to promote a deactivated state although A2A agonism may do this less than A1 agonism. These agonists were administered ICV into the lateral ventricles.

Materials and Methods

Drugs

Urethane (ethyl carbamate) was mixed in distilled water to yield a final concentration of 0.67g/ml. Adenosine and caffeine were dissolved in 0.9% saline to yield a final concentration of 100 nmol and 100 μM, respectively. These drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO). CGS 21680 and CPA were dissolved in 0.9% saline to yield a final concentration of 50 μM and 3μM, respectively. These drugs were purchased from Cayman Chemicals (Ann Arbor, MI). For systemic injections, a dose of 10mg/kg was used for both adenosine and caffeine.

General and Surgical Methodology

Male[1] Sprague Dawley rats (n = 31) weighing 250-320g were initially anesthetized using isoflurane (4% induction, 2-2.5% maintenance) to allow for implantation of a jugular catheter to administer urethane slowly until the rat reached surgical plane. Anesthetic plane was assessed by monitoring breathing rate and reflex withdrawal to hind-paw pinch. After the rats were secured into ear bars in a stereotaxic frame, body temperature was maintained at 37°C using a servo-controlled heating pad driven by rectal temperature (TR- 100, Fine Sciences Tools; Vancouver, BC, Canada). Before incision of the scalp, 0.5 ml of lidocaine (2%) was delivered subcutaneously across the midsagittal extent of the skull. After leveling the skull by ensuring that both bregma and lambda were at the same horizontal level, stereotaxic coordinates were measured from

[1] The majority of our previous work has taken place in male rats. Although females show similar brain state alternations (Whitten et al., 2009) under urethane, we have yet to undertake a more extensive comparison across sex.

bregma. Two recording sites were used: frontal cortex and hippocampus. The cortical site was positioned +2.8mm AP and +2.0 ML from bregma and -1.0 to -1.3mm ventral to the dural surface. The hippocampal site was positioned -3.5mm AP and -2.5 ML from bregma and -2.8 to -3.3mm ventral to dural surface. An additional burr hole approximately midway between the cortical and hippocampal sites was made to allow for a stainless-steel screw to be fixed to the skull. Bipolar recording electrodes were constructed from twisted Teflon-insulated stainless-steel wires (bare diameter 125 μm: A-M Systems Inc.) and implanted into the cortex and hippocampus.

Recording Procedures

Local field potential recordings made with bipolar, staggered electrodes were amplified differentially across contacts at a gain of 1,000 and filtered between 0.1 and 500 Hz using a differential AC amplifier (Model 1700; A-M Systems). Data were sampled at 1 kHz and digitized using a PowerLab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO) connected to a Windows 10 computer, and acquired using LabChart (AD instruments, Colorado Springs, CO).

Drug Administration

In some animals, peripheral injections of substances were made intravenously (i.v.) (through the PBS-cleaned urethane catheter) delivering 0.01ml of drug every 10 seconds. In other animals, bilateral targeting of the lateral ventricles (+0.8 AP and ±1.5 ML from bregma and -4.6 to -4.8 ventral to the surface of the skull) was undertaken in order to make intracerebroventricular (ICV) administrations. ICV administration ensured

that any effects observed were a result of modulation of centrally located adenosine receptors.

All ICV drug injections were made bilaterally into the lateral ventricles over a period of 5 minutes in volumes of 5 µl (1 µl per minute) using a 25 µl micro syringe (Hamilton Co; Reno, NV) connected via PE 50 tubing to a 30-gauge stainless steel cannula. Controlled and continuous infusions were made using a single syringe infusion pump (Fisherbrand, Model 78-01001; Pittsburg, PA). Injections were made to the right lateral ventricle first, followed by injection into the left ventricle. After delivery of drug to the right ventricle, the cannula was left in position for an additional two minutes to allow diffusion to take place before removal and implantation in the left ventricle.

Experimental Procedures

Baseline recordings were made for a period of 1-1.5 hours prior to injections to establish stable cycling between the deactivated and activated state. For peripheral injections, drugs were delivered via the jugular catheter. For ICV drug delivery, injections were made bilaterally into the lateral ventricles as described earlier. Recording of brain activity resumed for at least 1 hour after drug administration to allow for observations of cycling. Control injections and infusions used a similar volume of vehicle administered in the same way.

Data Analysis

EEG data was first examined and truncated using LabChart (AD instruments, Colorado Springs, CO) followed by analyses in MATLAB (version R2019a, MathWorks, Natick, MA). Spectrograms were constructed by computing power-spectral density

using the Welch periodogram method on sequential 30 second windows across the dataset, each separated by 10 seconds. Individual spectra used 6 second long, Hanning-windowed samples with a 2 second overlap across the 30 second data segment. Further analysis and visualization was performed in Origin (Microcal Software Inc.; Northampton, MA).

By extracting power at the SO (0.5-1.3Hz) and theta (3-5Hz) bandwidths, power fluctuations across time were monitored and plotted. As we have previously shown (Clement et al, 2006), both these bandwidths at the level of the neocortex and hippocampus, respectively, allow for characterization and assessment of deactivated and activated states, respectively, when power is high in either. NREM-like (deactivated) episodes were characterized based on the presence of high-amplitude, low-frequency cortical or hippocampal activity while REM-like (activated) episodes were classified based on the presence of low-amplitude, high-frequency cortical activity and by the presence of high hippocampal theta activity. By using a SO/theta band power ratio, sensitivity to state alternations could be enhanced. Bimodality of the amplitude histograms of these plots across time allowed us to determine thresholds (at the inter-modal trough) for state transitions from deactivated to activated and vice versa. This enabled us to calculate the duration of each activated state, deactivated state, and overall cycle period. One full cycle length was defined as the end of one deactivated state to the end of the next. The percent of activated vs. deactivated state was examined within each cycle using threshold crossings to determine if any changes occurred after drug administration. Changes in both the overall cycle length and the duration of activated/deactivated episodes allowed us to determine how any manipulation affected

state dynamics. The onset of drug effect was calculated as the amount of time taken to see an observable change from baseline cycling after bilateral injections were made. The duration of drug effect was calculated as the total time from onset of drug effect to drug washout and the return of cycling to baseline. Numeric averages were computed pre- and post-injection along with the SEM. Significance before and after manipulations was assessed using Student's t-test ($P < 0.05$).

Histology

To ensure the appropriate localization of cannulae to the lateral ventricles, histological examinations were conducted. Following experimental recordings, anesthetized rats were transcardially perfused (first with saline and later with 4% paraformaldehyde) and their brains were removed to allow for subsequent frozen section histology. The extracted brains were stored in 30% sucrose in 4% paraformaldehyde. Once sunk in this solution, the brains were subsequently frozen with compressed CO_2 and sliced at 60 μm with a rotary microtome (Leica 1320 Microtome; Vienna, Austria). Slices were then mounted onto gel-coated slides and dried for a minimum of 24 hours prior to being stained with thionin.

Results

As we have previously reported (Clement et al., 2008; Silver et al., 2021; Whitten et al., 2009), spontaneous and cyclic electroencephalographic alternations between deactivated (NREM-like) and activated (REM-like) patterns under urethane were highly stable and similar to those observed in natural sleep. Deactivated patterns consisted of high amplitude, low frequency SO (~1 Hz) activity in the cortex and concomitant low frequency activity in the hippocampus whereas activated patterns were characterized by the presence of rhythmic theta (~4 Hz) power in the hippocampus and low voltage, fast activity in the cortex **(Fig 2A-C). Figure 2A** shows a 5-second sample from long duration recordings in **Fig 2B** of LFP during the activated and the deactivated state from both the cortex and the hippocampus. In long-term recordings, alternations between these states were highly noticeable as fluctuations in the amplitude of raw LFP signals in the forebrain **(Fig 2B)**. By performing spectrographic analyses, we could show that the increase in amplitude of the LFP was associated with enhanced power in the SO band (~ 1 Hz) in both the neocortex and hippocampus which was denoted as the deactivated state **(Fig 2C)**. In the hippocampus, the activated state could also be tracked by monitoring the increase in power within the theta (~3-4 Hz) bandwidth. By extracting the 1 Hz power from these plots (indicated by the horizontal black dotted line in Fig 2C), we could track the alternations of the deactivated state by calculating a threshold level over which we could demarcate the start and end of each instance. As well, we could also track the duration of each alternation cycle by measuring the duration from one transition out of the deactivated state until the next. In some cases, we computed a ratio

of SO power to theta power to expand the range between the two states and facilitate the thresholding of state **(Fig 2D)**.

During control conditions and during the baseline period, we observed stable and consistent cycling between states (a minimum number of 3 cycles). This allowed us to then evaluate drug effects based on changes in the subsequent cycle duration and the percentage of deactivated patterns per cycle. In control conditions in which saline injections were made, brain state alternation dynamics remained unchanged as both the cycle duration and percentage of deactivated activity per cycle did not differ significantly after injection **(Figure 2E-F)**. Although the average percent deactivated per cycle increased slightly from 30.20% ± 3.59% to 32.04% ± 2.89%, this was not significant ($p=0.2017$) **(Fig 2E)**. The average cycle duration decreased from 11.07 ± 0.63 to 9.90 ± 1.21 minutes but this effect was not significant ($p=0.4083$) **(Fig 2F)**.

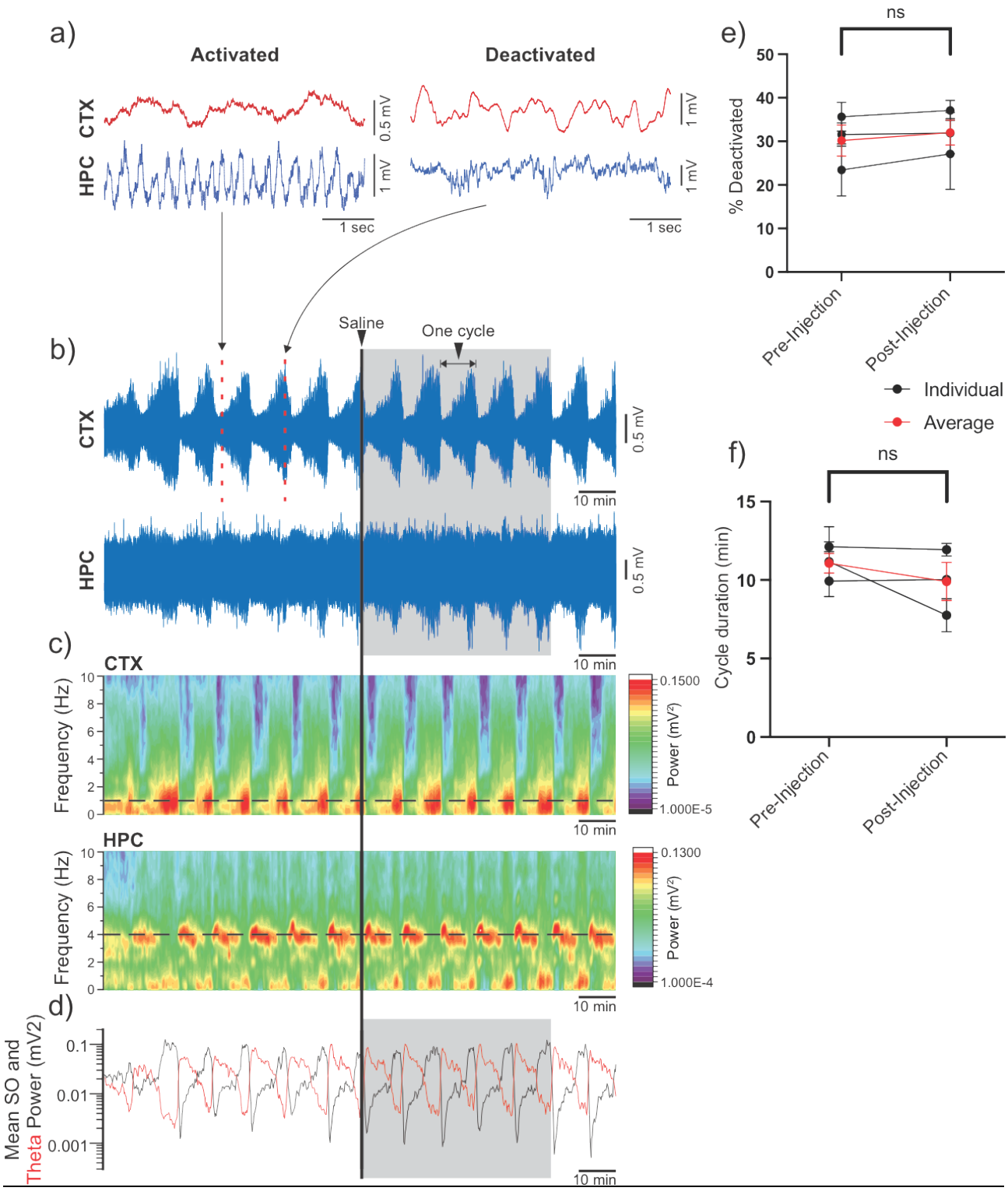

Figure 2. Urethane anesthetized rats displayed spontaneous and cyclic alternations in brain state that were unchanged before and after saline administration. **a)** 5-second

sample LFPs from the CTX and HPC during the activated and deactivated states. During the activated state, the CTX showed low voltage fast activity and the HPC showed prominent theta at ~4 Hz. During the deactivated state, there was a shift to low (~1 Hz) frequencies with an increase in overall power. **b)** Continuous EEG traces over a longer time scale demonstrated a regular and cyclic alternation of state as observed by the fluctuations in amplitude in the CTX and HPC. Vertical black line indicates saline injection. **c)** Spectrographic representation of cortical and hippocampal traces shown in c). **d)** Overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. **e)** and **f)** illustrate the percent deactivated and average cycle duration before and after saline administration, respectively. Total n=3.

IV administration of adenosine increases the deactivated state under urethane anesthesia

At an average latency of 3.33 ± 0.54 minutes following intravenous administration of 10mg/kg adenosine we observed an increase in the percentage of the deactivated state expressed across alternation cycles (n=5). This effect lasted for an average of 60.07 ± 9.56 minutes. As shown in the raw traces of cortical and hippocampal LFP in Figure 3A, there was an obvious but transient increase in the density of large amplitude slow activity characteristic of the deactivated state post-injection (see grey box in **Fig 3A)**. This effect was also seen as higher and longer-lasting power increases in the SO bandwidth (~1Hz) in the colour contour spectrograms for both neocortical and hippocampal sites. In contrast, there was a loss of power in the theta (~4Hz) bandwidth (corresponding to the activated state) in the hippocampal spectrogram post injection.

By tracking the SO power in the neocortex and the theta power in the hippocampus we observed no change in the brain-state alternation cycle durations but a definitive increase in the duration of the deactivated state and concomitant decrease in the activated state for each cycle across a period of time corresponding to approximately 4 cycles in this example (**Fig 3B-C)**. Across experiments, there was a statistically significant increase in the percentage of the deactivated state per cycle for a period corresponding to a minimum of 5 cycles following adenosine injection. On average, the percentage of deactivated states per cycle increased from 41.84% ± 6.06% to 74.10% ± 6.46% (p=0.0045) **(Fig 3D)**. Although the average cycle duration also increased on average from 10.27 ± 0.40 to 13.03 ± 1.86 minutes, this effect was not significant (p=0.2673) **(Fig 3E)**.

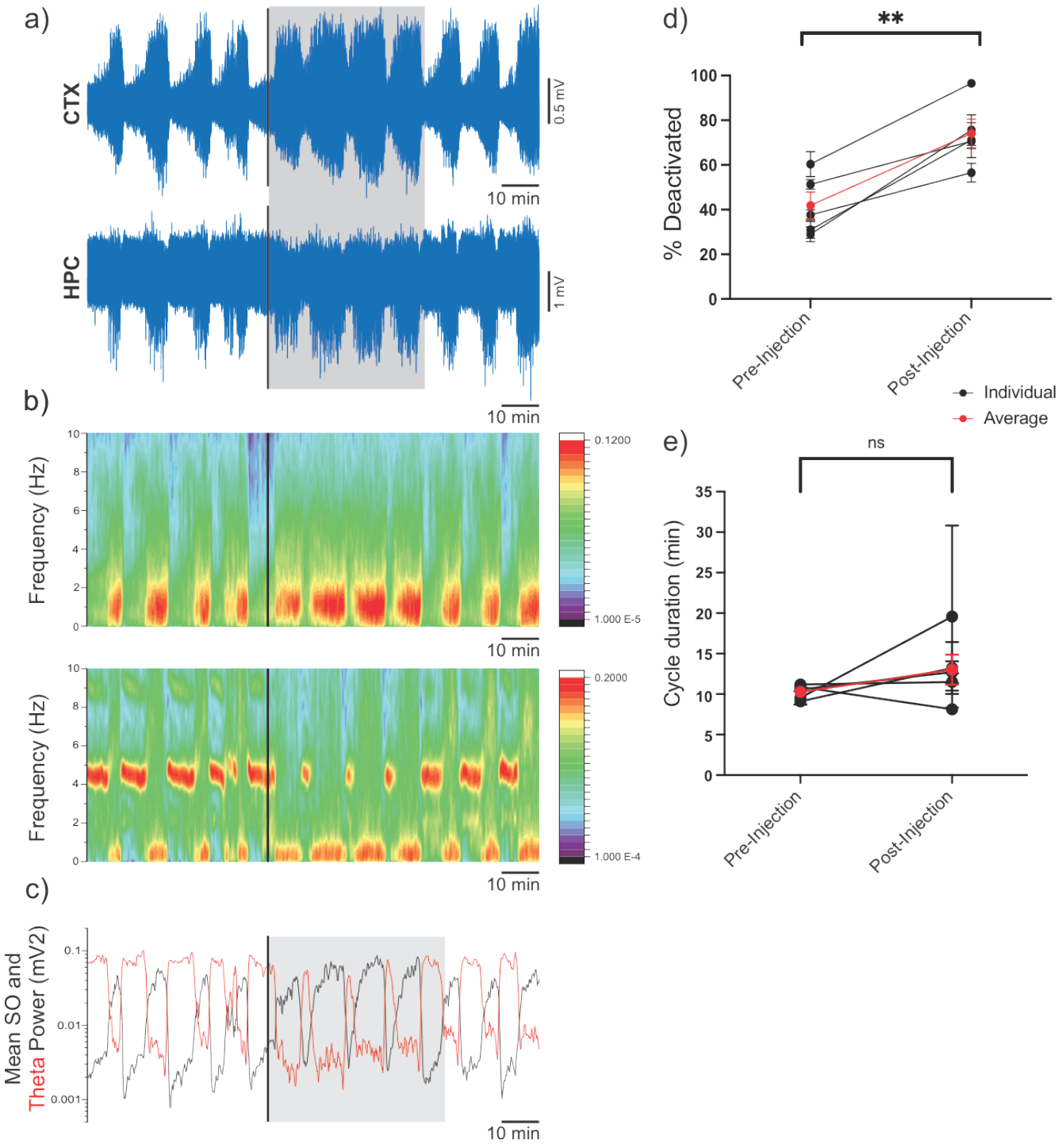

Figure 3: Administration of adenosine IV increases the deactivated period. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces. **b)** spectrographic representation of cortical and hippocampal traces shown in a). **c)** displays an overlay of

mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The adenosine effect is highlighted in grey. **d)** and **e)** illustrate the percent deactivated and average cycle duration before and after adenosine administration, respectively. Total n = 5.

IV administration of caffeine decreases the deactivated state under urethane anesthesia

In contrast to the effects with adenosine, intravenous caffeine administration caused an overall decrease in the deactivated state (n=5) **(Fig 4)**. This effect occurred at a greater latency than that for adenosine, averaging 19.69 ± 10.97 minutes post-injection with a mean duration effect of 68.33 ± 5.84 minutes. Raw traces of cortical and hippocampal LFP indicated an noticeable decrease in the density of large amplitude slow activity post-injection **(Fig 4A)**. By again tracking SO and theta power we observed a decrease in the deactivated state post caffeine injection **(Fig 4B)**. There was a statistically significant decrease in the percentage of time spent in the deactivated state cycle-by-cycle after caffeine administration. On average, the percent deactivated decreased from 43.88% ± 3.33% to 32.95% ± 1.96% (p=0.0281) **(Fig 4C)**. Across experiments, average cycle duration increased from 10.43 ± 0.46 to 15.533 ± 2.72 minutes, but this effect was not found to be significant (p=0.1205) **(Fig 4D)**. However, in 2 of the 5 experiments in this drug group, there was a significant increase in cycle duration after injection (p=0.003; p=0.0116).

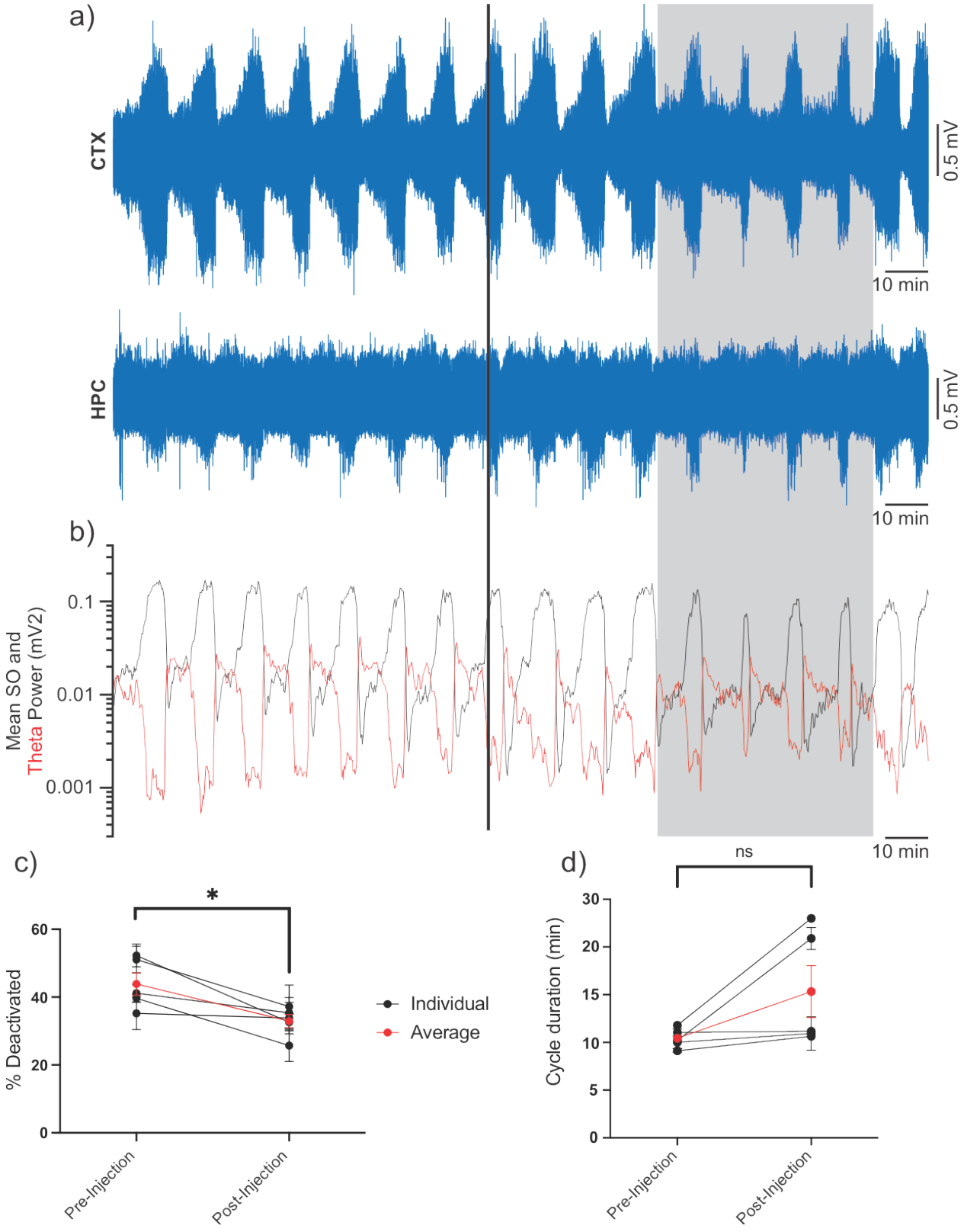
a)
CTX
0.5 mV
10 min
HPC
0.5 mV
10 min
b)
Mean SO and
Theta Power (mV2)
0.1
0.01
0.001
10 min
c)
*
% Deactivated
60
40
20
0
Pre-Injection
Post-Injection
Individual
Average
d)
ns
Cycle duration (min)
30
20
15
10
5
0
Pre-Injection
Post-Injection

Figure 4: Administration of caffeine IV decreases the deactivated period. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces from baseline to the end of the experiment. **b)** displays an overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The vertical black line through a) and b) denotes injection time of caffeine. The effect of caffeine is highlighted in grey. **c)** and **d)** illustrate percent deactivated cycle-by-cycle and mean cycle duration before and after caffeine administration, respectively. Total n=5.

Co-administration of adenosine and caffeine IV increases the deactivated state initially with an increase in the activated state later

We attempted to challenge the adenosine effect by co-administration of both it and its nonspecific antagonist caffeine. What we observed was a biphasic response starting with a limited increase in the deactivated state, which was then truncated by a subsequent decrease (n=4) **(Fig 5A-B)**. The latency to the increase was on average was 5.71 $\pm$ 5.07 minutes with a mean effect duration of 60.19 $\pm$ 15.25 minutes. The average latency to the reduction in deactivated patterns was 66.15 $\pm$ 15.42 minutes with a mean effect duration of 84.79 $\pm$ 20.75 minutes. An increase in large amplitude slow wave activity followed by a subsequent decrease can be observed in the long duration EEG traces of the cortex and hippocampus shown in **Fig 5A**. By tracking SO and theta power post-injection, we observed an initial increase in SO power with a concomitant decrease in theta power followed by the opposite effect **(Fig 5B)**. The increase in deactivated patterns went from 33.0% $\pm$ 1.70% to 78.5% $\pm$ 10.07% **(Fig 5C)**. This effect was observed to be statistically significant (p=0.0124). Although the subsequent loss of this

increase in deactivated patterns was not significantly different from baseline conditions (33.0% ± 1.70% to 24.9% ± 5.49%; p=0.3007), it was a significant drop from the initially increased values (p=0.0319) **(Fig 5E)**.

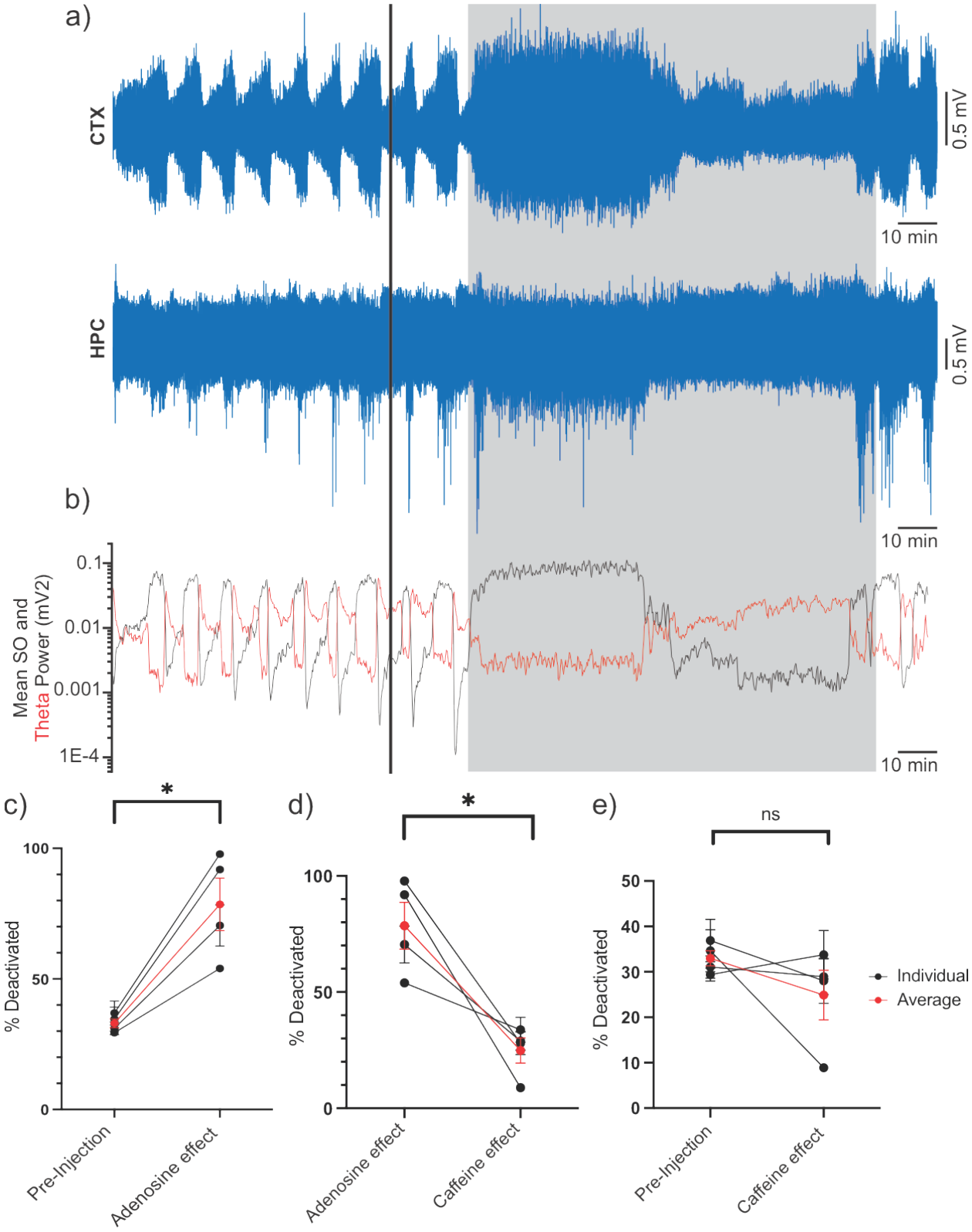
a)
CTX
0.5 mV
10 min
HPC
0.5 mV
10 min
b)
Mean SO and
Theta Power (mV2)
0.1
0.01
0.001
1E-4
10 min
c)
*
% Deactivated
100
50
0
Pre-Injection
Adenosine effect
d)
*
% Deactivated
100
50
0
Adenosine effect
Caffeine effect
e)
ns
% Deactivated
50
40
30
20
10
0
Pre-Injection
Caffeine effect
Individual
Average

Figure 5: Co-administration of adenosine and caffeine increases the deactivated state initially followed by a decrease in the deactivated state. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces from baseline to the end of the experiment. **b)** displays an overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The vertical black line through a) and b) denotes injection time. Effect of adenosine and then caffeine is highlighted in grey. **c)** demonstrates the comparison of the percent deactivated during baseline and during the effect of adenosine. **d)** illustrates change in percent deactivated between the adenosine and caffeine effect. **e)** shows the comparison of the percent deactivated during baseline and the effect of caffeine. Total n=4.

ICV administration of adenosine increases the deactivated state under urethane anesthesia

Similar to what was observed with IV delivery of adenosine, ICV injections of adenosine also promoted the deactivated state (n=4) **(Figure 6)**. This increase occurred with a slightly slower latency but was still fairly rapid, averaging 9.20 $\pm$ 3.38 minutes. It also tended to last longer, having a mean duration of 83.73 $\pm$ 5.58 minutes. As with i.v. adenosine there was an increase in the density of large amplitude slow rhythms in both cortical and hippocampal LFP recordings which was also confirmed with power measures in the SO band. Also similar to i.v. adenosine, theta band power showed a marked decrease following infusions as well **(Fig 6A-B)**. Unlike systemic adenosine, however, there was significant slowing of the alternation cycle duration following this manipulation from 11.01 $\pm$ 0.84 to 18.08 $\pm$ 1.76 minutes (p=0.0327) **(Fig 6E)**. Given this

slowing, the cycle-by-cycle analysis of deactivated state proportions was not possible. Thus, we compared the raw durations of both the activated and deactivated states in a 1-hour window pre-infusion and during the drug effect. There was a significant decrease in the activated state from 6.39 ± 0.98 to 2.54 ± 0.42 minutes on average (p=0.0287) **(Fig 6C)** while the average deactivated state duration increased significantly from 4.60 ± 0.81 to 15.53 ± 2.04 minutes (p=0.0132) **(Fig 6D).**

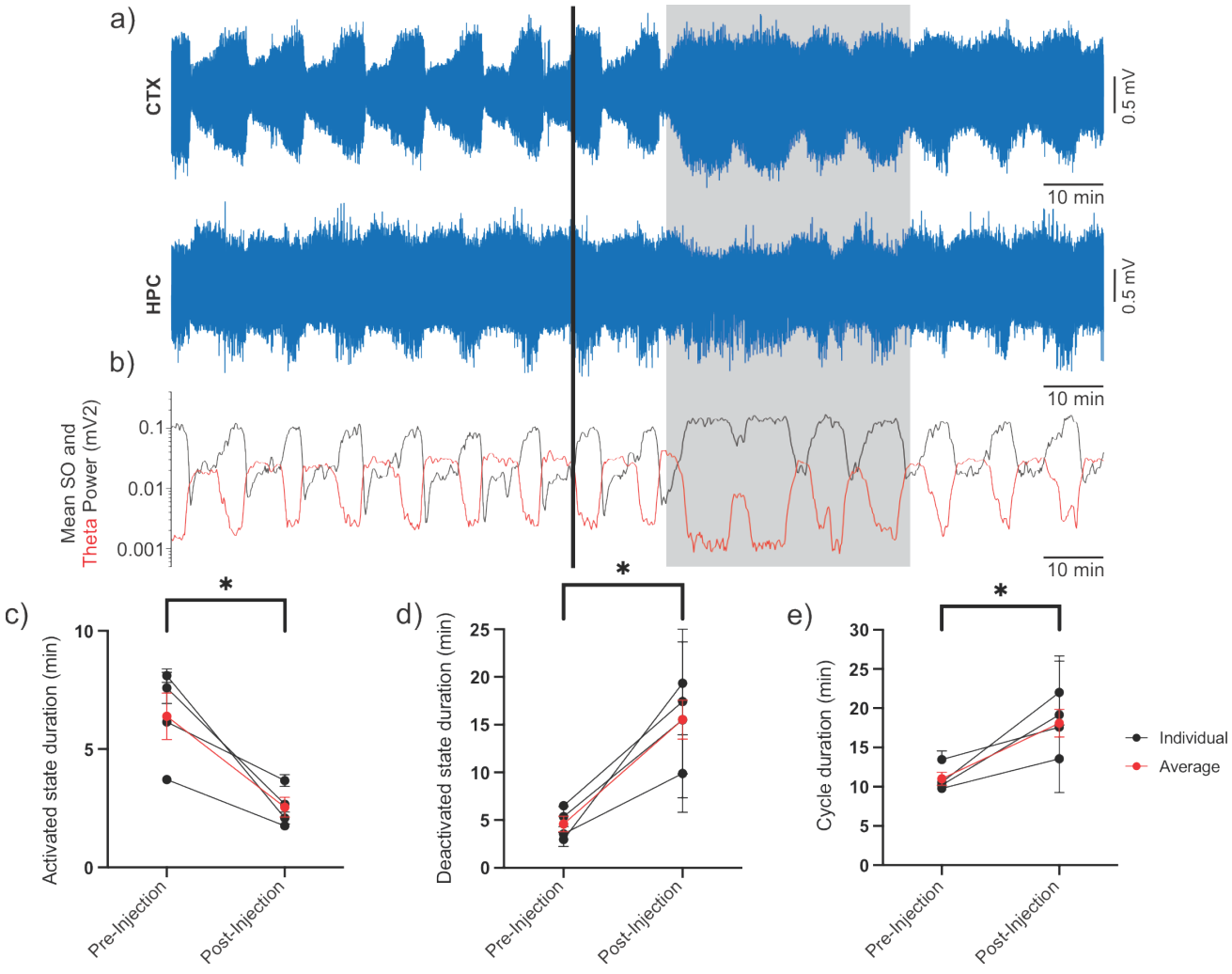

Figure 6: ICV application of adenosine increases duration of the deactivated state. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces from baseline to the end

of the experiment. **b)** displays an overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The adenosine effect is highlighted in grey. **c)** and **d)** illustrate the duration of the activated and deactivated state before and after adenosine administration, respectively. **e)** represents the change in cycle duration after drug administration. Total n=4.

ICV administration of caffeine decreases the deactivated state under urethane anesthesia

ICV administration of caffeine, in contrast to adenosine, resulted in an overall decrease in the deactivated state (n=4). This effect occurred at an average greater latency than that for adenosine, averaging 11.15 ± 1.44 minutes post-injection with a mean effect duration of 40.38 ± 4.18 minutes. **Figure 7A** shows an obvious decrease in the amount of time spent in the deactivated state and an increase in the density of the activated state following this manipulation. This decrease in the deactivated state was also noticeable when tracking SO and theta power post caffeine injection **(Fig 7B).** There was a statistically significant decrease in the percentage of time spent in the deactivated state on a cycle-by-cycle basis when compared to baseline. On average, the percent deactivated decrease from 43.78% ± 4.94% to 22.33% ± 7.65% (p=0.0063) **(Fig 7C)**. Across experiments, the average cycle duration increased from 11.13 ± 1.69 minutes to 21.80 ± 5.88 minutes but this increase was not significant (p=0.0948) **(Fig 7D)**.

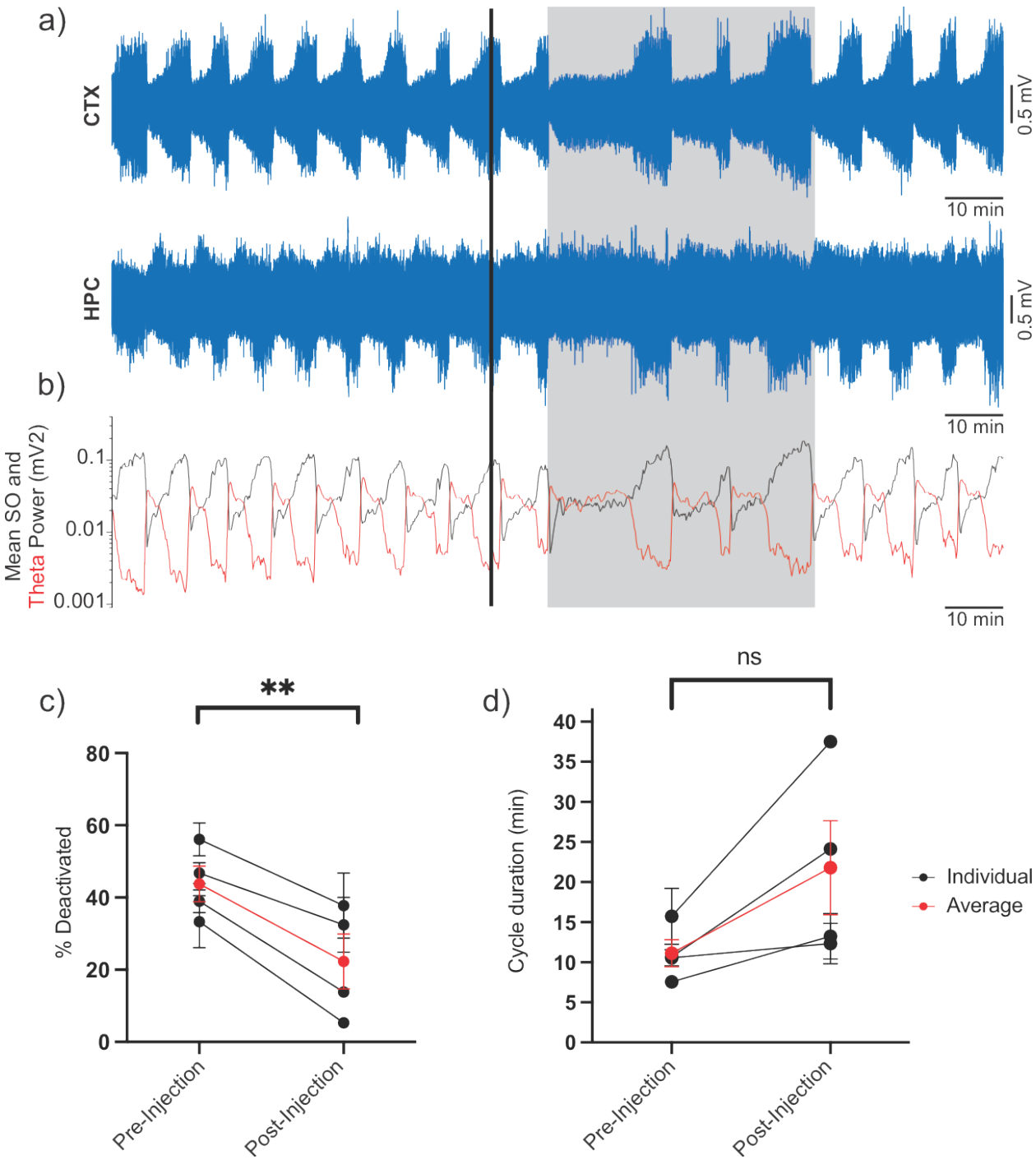

Figure 7: Administration of caffeine ICV decreases the deactivated period. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces from baseline to the end of the experiment. **b)** displays an overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The vertical black line through a) and b) denotes injection time of

caffeine. The effect of caffeine is highlighted in grey. **c)** and **d)** illustrate percent deactivated cycle-by-cycle and mean cycle duration before and after caffeine administration, respectively. Total n=4.

ICV administration of CGS increases the deactivated state under urethane anesthesia

Administration of the A2A agonist CGS 21680 led to an increase in the deactivated state (n=3) **(Fig 8).** The average latency to drug effect was 19.47 ± 1.24 minutes with an average effect duration of 81.61 ± 6.28 minutes. The promotion of the deactivated state can be observed both in the long duration cortical and hippocampal LFPs via an increase in the density of SO activity as well as by the noticeable decrease in the mean theta power **(Fig 8A-B)**. On average, the percent deactivated increased from 36.5% ± 1.72% to 71.2% ± 2.53% across all experiments **(Fig 8C)**. The change in deactivated percentage pre and post injection was statistically significant (p=0.0009). The average change in cycle duration increased from 10.28 ± 1.16 to 11.06 ± 1.57 minutes but this change was not statistically significant (p=0.2210) **(Fig 8D)**.

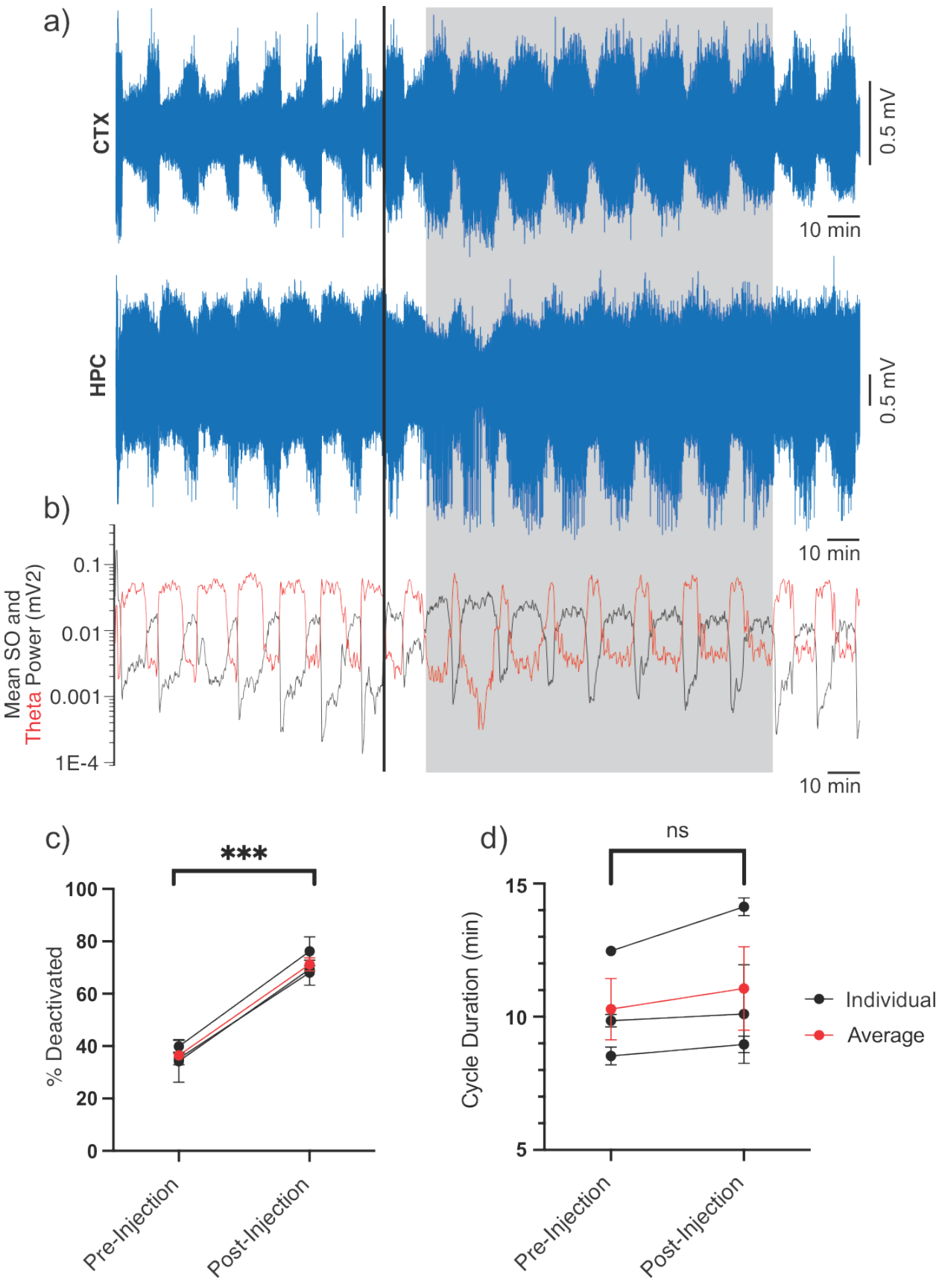
a)
CTX
0.5 mV
10 min
HPC
0.5 mV
10 min
b)
Mean SO and
Theta Power (mV2)
0.1
0.01
0.001
1E-4
10 min
c)
***
% Deactivated
100
80
60
40
20
0
Pre-Injection
Post-Injection
d)
ns
Cycle Duration (min)
15
10
5
Pre-Injection
Post-Injection
Individual
Average

Figure 8: CGS 21680 increases the deactivated state. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces from baseline to the end of the experiment. **b)** displays an overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The vertical black line through a) and b) denotes injection time of CGS 21680. Effect of CGS 21680 is highlighted in grey. **c)** and **d)** illustrate percent deactivated cycle-by-cycle and overall cycle duration before and after CGS 21680 administration, respectively. Total n=3.

ICV administration of CPA increases the deactivated state under urethane anesthesia

Central administration of the A1 agonist CPA resulted in rapid and massive changes in the dynamics of forebrain states (n=3). Overall, this was manifest as an almost permanent switch to the deactivated state which was blatantly obvious in the raw cortical LFP recordings. However, but also striking, there was a consistent and massive drop off of signal amplitude in the hippocampus **(Fig 9)**. Indeed, cycle alternations were effectively eliminated after CPA administration and in all examples, cortical activity was uniformly characteristic of the deactivated state **(Fig 9A-B)**. The effect was present at an average latency of 3.17 $\pm$ 0.54 minutes with a mean effect duration of 133.83 $\pm$ 17.16 minutes. In all 4 experiments, true cycling never returned even after hours following the infusion. As shown in the raw traces of cortical LFP, there was a robust increase in the density and amplitude of large amplitude slow wave activity characteristic of the deactivated state post-CPA, whereas in hippocampus there was a marked decrease in overall signal strength **(Fig 9A)**. When comparing mean SO (~1 Hz) and mean theta (~4

Hz) power, there was an obvious and near-permanent increase in SO with a concomitant decrease in theta power soon after CPA administration **(Fig 9B)**. Although the drug effect did not washout over the course of our experiments, some return of preliminary cycling was observed at longer latencies (85.96 $\pm$ 4.36 minutes) **(Fig 9A-B)**. On average, percent deactivated increased from 32.39% $\pm$ 9.88% to 91.45% $\pm$ 3.98% **(Fig 9C)**. This increase in percent deactivated was statistically significant (p=0.0134).

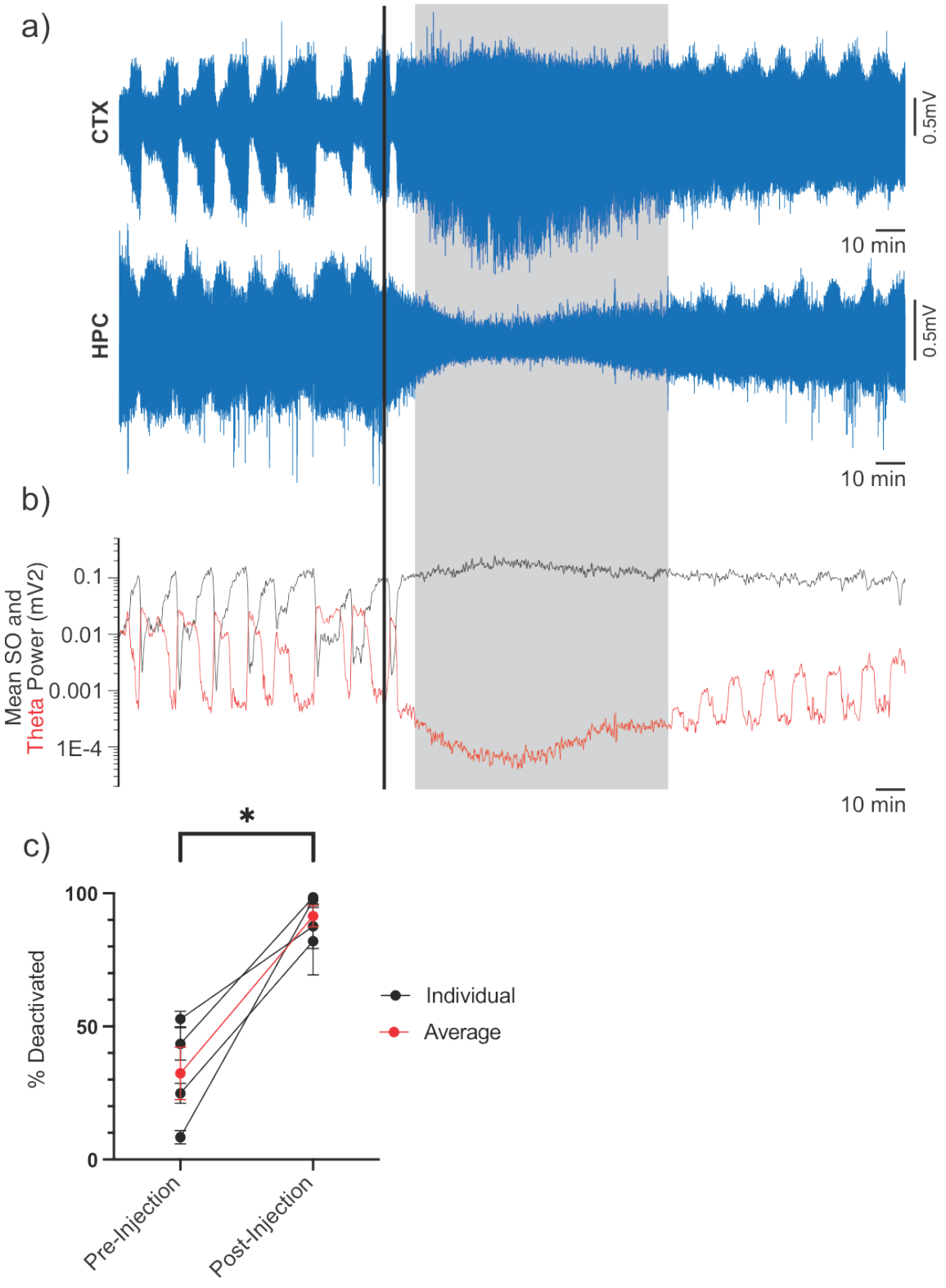

Figure 9: CPA increases the deactivated state and abolishes cycling. **a)** shows cortical (top) and hippocampal (bottom) raw EEG traces from baseline to the end of the

experiment. **b)** displays an overlay of mean SO (~1 Hz) and mean theta (~4 Hz) power as a function of time. The vertical black line through a) and b) denotes injection time of CPA. The effect of CPA is highlighted in grey. **c)** illustrates percent deactivated cycle-by-cycle. Total n=3.

Discussion

The purpose of this work was to critically evaluate the urethane model of natural sleep. Previous work from our lab has shown that urethane is a useful anesthetic to model sleep-state dynamics (Clement et al., 2008; Hauer, Pagliardini, & Dickson, 2019; Pagliardini et al., 2013; Pagliardini, Greer, Funk, & Dickson, 2012b; Silver et al., 2021; Ward-Flanagan & Dickson, 2019; Whitten et al., 2009). The ability of urethane to initiate an unconscious and anesthetised state that imitates natural sleep in terms of its ability to allow spontaneous brain state alternations makes it an unparalleled model for sleep itself. Here, we have provided more evidence for the neurobiological overlap of urethane and natural sleep by the similar actions of adenosinergic agents.

Sleep – The final frontier of brain research

The evolutionary advantage or reason behind why we sleep remains a fascinating mystery. Both short-term and long-term disturbances in the quality or quantity of sleep are known to be problematic and detrimental to normal physiological functioning and health. There are many factors that control sleep but largely speaking, sleep is regulated via circadian and homeostatic mechanisms. Given that adenosine is a fundamental constituent of the homeostatic control of sleep, improved understanding of adenosine and its involvement in sleep drive can potentially help us to better understand the function of sleep. Studying sleep itself, however, is challenging as it is easily disrupted making controlled experimentation difficult. Research has implicated anesthetics as a potential avenue to study sleep given the neurobiological parallels between the unconsciousness generated by these states (Adapa, 2017; Mashour & Pal, 2012; Ward-Flanagan & Dickson, 2019). As indicated by the results of this study, the high degree of

similarity in brain state alternations between urethane anesthesia and natural sleep made it a superlative anesthetic to evaluate adenosinergic action.

Adenosine actions under urethane anesthesia parallel those in natural sleep

As reviewed previously, adenosine has been suggested to be a potent sleep factor and essential for maintaining sleep homeostasis. Therefore, we wanted to examine if the effects of adenosine in natural sleep could be replicated under urethane anesthesia. Previously, numerous studies have reported that activation of adenosine receptors (either A1 or A2A) increases NREM sleep (Benington et al., 1995; Hong et al., 2005; Li et al., 2020; Oishi et al., 2008; Scammell et al., 2001).

Collectively, my work demonstrated that adenosine, as well as A1 and A2A receptor agonists, promote the deactivated (or non-REM-like) state under urethane as well. Conversely, antagonizing adenosine receptors using caffeine (both centrally and peripherally) decreased in the deactivated state. This is also similar to the impairment of SWS by caffeine in natural sleep (Schwierin et al., 1996). Cumulatively, these findings corroborate our initial hypothesis. Moreover, our parallel results under urethane further supports this anesthetic as a model for natural sleep.

When comparing our findings between peripheral and central administration of adenosine, there were some curious differences. As reported, the alternation cycle was elongated by ICV adenosine but not with IV applications. This could perhaps be a result of strong agonism of A1 receptor rich areas near the lateral ventricles such as the hippocampus. Additionally, the effect of adenosine lasted longer when it was given centrally. This finding suggests that central activation of adenosine receptors *specifically* may more robustly promote the deactivated state and be more critical for sleep

homeostasis. It has been reported that increases in extracellular adenosine within specific brain areas (such as the basal forebrain) is crucial for somnogenic effect. Therefore, central activation of adenosine receptors may be more essential for overall control of sleep need. Another possibility, however, is that when injected peripherally directly into the blood stream, adenosine would be cleared more rapidly since blood likely has a very different compliment of adenosine enzymes and transporters. Interestingly, although the decrease in slow-wave density caused by ICV caffeine was greater than that observed with IV applications, the duration of the latter effect lasted longer.

Fascinatingly, the co-administration of adenosine and caffeine revealed a somewhat surprising biphasic effect characteristic of both drugs instead of an immediate antagonism of the non-REM promoting action of adenosine. Our results from administration of adenosine and caffeine alone showed that the latency to the adenosine effect was much faster than the latency to caffeine effect. Perhaps it is the case that because of this faster latency to the adenosine effect, the deactivated state was seen soon after co-administration with a later decrease brought on by the eventual antagonism of adenosine receptors by caffeine. Previously, It has been reported that an increase in adenosine and A1 activation as a result of prolonged wakefulness can lead to an increase in A1 receptor mRNA (Basheer et al., 2001; Porkka-Heiskanen, Kalinchuk, Alanko, Urrila, & Stenberg, 2003). However, this is likely not a possible explanation for the effect we observed since this upregulation of mRNA takes several hours. It can be predicted that if a higher concentration of caffeine was used, this presumed effect of adenosine and the deactivated state would be challenged more robustly leading to a

decreased in this state or perhaps no effect at all. Additionally, it can be predicted that given the quick latency of the adenosine effect, the effect of caffeine could be interrupted or weakened if adenosine was administered again.

Our results also showed that the effects of A1 and A2A agonism using the respective receptor agonists CPA and CGS 21680 were very different. Overall, both agonists increased the deactivated state, but A1 receptor activation using CPA practically eliminated the activated state, and did it for a dramatically extended period of time. Therefore, these results further support the concept that not only does adenosine action at its different receptor subtypes promote the deactivated (NREM-like) state, but that this is done in different ways. Both A1 and A2A agonism in natural sleep promotes SWS thus our overall results from these agonists (with regard to an increase in the deactivated state) are consistent with what has been reported (Benington et al., 1995; Hong et al., 2005; Li et al., 2020; Oishi et al., 2008).

Interestingly with A1 receptor agonism using CPA, there was a substantial drop in the signal amplitude for the hippocampus. A1 receptors are highly concentrated in the hippocampus and their activation has an overall inhibitory effect on neuronal excitability which could be a possible explanation for the drop in hippocampal EEG amplitude. Previous work has shown that A1 receptor activation can decrease excitatory synaptic transmission in the rat hippocampus (Dunwiddie & Fredholm, 1984). Moreover, bath application of rat hippocampal slices with L-PIA (another A1 agonist) resulted in a depression of field responses in the hippocampus (Dunwiddie, Basile, & Palmer, 1984).

Together, our findings further support the idea that both A1 and A2A receptors are involved in sleep promotion but that they are likely playing different roles as

previously suggested by Lazarus et al., (2019). Interaction with A1 receptors has been suggested to be essential for sleep maintenance and expressing sleep need (sleep homeostasis) whereas A2A receptors have been proposed to be crucial for sleep gating and suppressing arousal (Lazarus et al., 2019). Action of adenosine at A1 receptors has been suggested to be directly permissive for SWA (Lazarus et al., 2019) and CPA administration has been shown to increase NREM sleep and SWA in a way that is similar to what is observed with sleep deprivation (Benington et al., 1995). Instead, A2A receptor activation with CGS 21680 has been suggested to promote sleep by inhibiting the activity of arousal centres such as the TMN (Hong et al., 2005; Scammell et al., 2001). If A1 receptors are more crucial for sleep homeostasis, then it makes sense that the increase in the deactivated period was observed to be larger with CPA administration. The site of injection in this case (ICV) could also have added to this effect since neighbouring areas include the hippocampus which is rich in A1 receptors.

Adenosine is a crucial homeostatic regulator of sleep

The involvement of adenosine and its analogs in promoting and maintaining sleep is crucial to developing a better understanding of sleep itself. As an endogenous target, adenosine's involvement in sleep-wake circuitry is paramount and can have numerous implications. The neurobiological mechanism and targets by which adenosine exerts its somnogenic effect remain unclear and contested. A variety of regions have been implicated in the somnogenic effect of adenosine including the basal forebrain, nucleus accumbens, VTA, OT, and TMN. However, a deeper understanding of how adenosine promotes sleep *globally* is crucial. Therefore, in order to continue delineating adenosinergic involvement in sleep, a unified model encompassing the homeostatic (A1)

and arousal (A2A) component is compulsory. Nonetheless, adenosine is a key homeostatic regulator of sleep and an improved understanding of its role in sleep can ultimately lead to an enriched awareness of sleep function.

Urethane as an unparalleled model of natural sleep

The purpose of my work was to determine the similarity of adenosinergic action in urethane anesthesia and natural sleep. My work here continues to validate the urethane model of natural sleep and reiterates its usefulness in modeling sleep-state dynamics. The unique ability of urethane to mimic natural sleep without compromising state alternations makes it a powerful anesthetic which can be used to investigate the influence of a variety of compounds on sleep state dynamics. We have previously shown that urethane anesthesia at a surgical plane produces the same EEG alternations between activated and deactivated brain states which are highly similar to those seen in natural sleep (Clement et al., 2008; Pagliardini et al., 2013; Silver et al., 2021; Ward-Flanagan & Dickson, 2019; Whitten et al., 2009). This, along with other commonalities makes urethane anesthesia an ideal model for both sleep and sleep-state dynamics (Clement et al., 2008; Pagliardini et al., 2012a; Ward-Flanagan & Dickson, 2019; Whitten et al., 2009). Furthermore, urethane anesthesia allows for controlled experimentation and long duration electrophysiological recordings which can be challenging, if not impossible, under natural sleep. Here, not only do we further validate the urethane model, but we also illustrate that adenosinergic control of brain state can be replicated under urethane. My results confirm what has been reported in natural sleep in that adenosine and its agonists increase NREM sleep whereas antagonists decrease NREM. Altogether, the replication of these results under urethane anesthesia makes a

compelling argument for why the urethane model should continue to be used to investigate sleep-state dynamics. Moreover, the urethane model can have meaningful implications for drug discovery in sleep disorders.

There are some limitations to this work that are worth mentioning. Firstly, it is important to note that although anesthesia is commonly referred to as “being put to sleep” and may appear similar to sleep behaviourally, they are two different states of unconsciousness. Urethane is unique in that it mimics sleep very closely; however, it is not a complete model because characteristic phasic features of REM sleep such as rapid eye movements are not observed under urethane. Another limitation is that these experiments are terminal due to the carcinogenic nature of urethane. One possible solution to this would be to use the anesthetic chloral hydrate which has been shown to have the same advantages without the need for terminal experiments (Ward-Flanagan, Lo, Clement, & Dickson, 2022). Secondly, this study included only male rats. Although neurobiological differences between male and female rats are worth considering, previous work from our lab has found that brain state alternations under urethane in male rats were also apparent in female rats (Whitten et al., 2009). Thus, it can be reasonably predicted that the effects observed in this study would be consistent in female rats. However, I recognize that we cannot say this for certain and it is imperative that future research examine the effect of these compounds in female rats. Moreover, although it was beyond the scope of this project, another potential limitation is the inability to know where these drugs were acting or what population of neurons were targeted. It will be crucial and beneficial for future research to investigate this, especially given receptor specific effects of adenosine. Future studies should examine the effects of these drugs

by conducting dye experiments. Mixing drugs with dye prior to injecting ICV would allow for the examination of what specific areas are implicated in the observed effects. Also, c-FOS studies could be conducted to evaluated what populations of cells are activated specifically when drugs are administered. Furthermore, future work should investigate adenosinergic involvement in sleep by making use of positive allosteric modulators (PAMs) similar to what has been done by Korkutata et al. (2019). PAMs bind to the orthostatic site and enhance the activity of the ligand only when the ligand is present (Korkutata et al., 2019). Thus, PAMs would more closely imitate the endogenous physiological activity of adenosine than an agonist.

Conclusion

Progress in understanding the neurobiology of sleep requires adequate models that allow for manipulations that might otherwise be unethical or disrupt the very state they are intended to study. Therefore significance of this research lies in demonstrating further pharmacological similarity between urethane anesthesia and sleep (Clement et al., 2008; Ward-Flanagan & Dickson, 2019). Having a validated anesthetic model of sleep will help to provide further insight into brain states during natural sleep, and perhaps, other forms of unconsciousness. As well, and also importantly, investigating adenosine effects on brain state can have important implications for purinergic signaling related to unconsciousness. Given that sleep is an important health factor, a better understanding of the mechanisms that trigger and maintain this state is paramount. This research has the potential to impact the physiological and mental health of individuals that may be suffering from sleep-related illnesses. An added benefit of examining adenosinergic modulation of sleep-related states is that it gives us a natural and endogenous molecule to promote as opposed to more typical pharmacological interventions. This may have profound implications for rethinking and reinventing the way that sleep disorders are treated which will perhaps lead to better and more personalized therapy for individual patients as well as an overall increase in the quality of life of patients in general.

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