

# Acute and chronic sleep restriction: Effects of caffeine on the EEG and heart rate during sleep, and waking attention

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***ACUTE AND CHRONIC SLEEP RESTRICTION: EFFECTS OF  
CAFFEINE ON THE EEG AND HEART RATE DURING SLEEP,  
AND WAKING ATTENTION.***

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES  
(Dr. sc. ETH Zurich)

presented by

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2024





*Dedicated to my daughter.*

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## Summary:

The widespread role of caffeine in modern society cannot be overstated, with millions of individuals relying on it to navigate the difficulties of a busy lifestyle. Despite the common usage of caffeine, its effects on sleep physiology and cognition have not yet been fully explored. This dissertation aims to fill some of these gaps by investigating how caffeine affects cognitive performance during sleep restriction in caffeine sensitive subjects, by examining the concentration dependent effects of caffeine on sleep and cardiac autonomic activity and potential in combating sleep inertia in the morning via a newly developed caffeine release formula.

In the first chapter we will look at the general link between sleep physiology, sleep-wake cycle, the importance of adenosine in sleep modulation and the consequent ramifications on cognitive and physiological functions after caffeine consumption. The consumption of caffeine, predominantly via coffee, is historically associated with the attenuation of sleepiness and the enhancement of vigilance. Research scrutinizes the variable responses to caffeine, influenced by different formulations, dosages, and genetic polymorphisms in adenosine receptors and caffeine metabolizing enzymes. We will look at a historical and scientific perspective on sleep research, contemporary EEG methodologies and its role in elucidating cerebral function during sleep. We will look at the importance of sleep ranging from cognitive aspects like memory consolidation to housekeeping roles involving neural repair, metabolite clearance and restorative process. Introduce various sleep stages, each characterized by distinct polysomnographic (PSG) patterns, specific physiological functions and lay the ground for the exploration of the complex interrelations between sleep, caffeine consumption, and the impact on cognitive and physiological parameters. In the second chapter we will explore the effectiveness of coffee in mitigating the cognitive decline associated with sleep restriction in individuals with a genetic predisposition to caffeine sensitivity. Using a double-blind design, this study compares the cognitive performance of participants consuming regular and decaffeinated coffee over five nights of restricted sleep. The findings revealed that while both groups reported increased subjective sleepiness, the group that consumed regular coffee experienced significantly better performance in sustained and selective attention, as well as executive control tasks. Importantly, these beneficial effects ceased after a recovery night, suggesting the impermanence of caffeine's efficacy.

In the third chapter we dive deeper into the physiological aspects of caffeine consumption, specifically focusing on its impact on EEG and cardiac autonomic activity during sleep. We probed blood plasma in regular time intervals to quantify caffeine concentration, alongside standard polysomnography and found that caffeine dose-dependently reduced delta activity and heart rate during non-REM sleep. Moreover, caffeine increased high-frequency spectral power in heart rate variability (HR-HRV), indicating elevated parasympathetic activity. This study establishes a quantitative concentration-effect relationship between caffeine, EEG delta power, and cardiac autonomic activity.

In the fourth chapter we examine caffeine's efficacy in combating sleep inertia, a state of grogginess and impaired vigilance immediately upon awakening. Utilizing an innovative, delayed, pulsatile-release caffeine formulation, this study showed significant improvements in sleep-to-wake transition among sleep-restricted volunteers. When compared to a placebo, this caffeine formulation accelerated reaction time on psychomotor vigilance tasks, improved positive and

negative affect scores, prolonged the cortisol awakening response, and delayed nap sleep latency.

Collectively this thesis sheds light on caffeine's role in moderating sleep and cognitive performance. From ameliorating the negative effects of sleep restriction to modulating cardiac and brain physiology during sleep, caffeine appears to offer a solution in a time and concentration dependent manner, i.e. the benefits and drawbacks of caffeine are highly contingent on its dosage, timing, and individual physiological factors, such as genetic predisposition to caffeine sensitivity. Furthermore, this work opens new avenues for developing specialized caffeine formulations, providing targeted relief from specific sleep-related issues like sleep inertia.

This dissertation aims to illuminate the complex relationship between caffeine, sleep, cognitive performance, genetic polymorphism and physiological response. It provides compelling evidence that caffeine can mitigate the cognitive and physiological impairments caused by sleep restriction and sleep inertia. These insights may help optimize the use of caffeine to enhance performance and well-being in modern society, especially in sleep-restricted conditions.

## Zusammenfassung:

Die allgegenwärtige Rolle von Koffein in der modernen Gesellschaft kann nicht genug betont werden, da sich Millionen von Menschen darauf verlassen, um die Anforderungen eines modernen Lebensstils zu bewältigen. Trotz der Allgegenwart von Koffein sind seine unterschiedlichen Auswirkungen auf die Schlafphysiologie und Kognition noch nicht vollständig erforscht. Diese Dissertation hat das Ziel, einige dieser Lücken zu schließen, indem untersucht wird, wie Koffein die kognitive Leistungsfähigkeit während des Schlafmangels bei koffeinempfindlichen Personen beeinflusst, indem die konzentrationsabhängigen Auswirkungen von Koffein auf Schlaf, Herzfrequenz und autonome Aktivität untersucht werden und das Potenzial zur Bekämpfung von Schlafträgheit am Morgen mit einer neu entwickelten Koffein-Freisetzungformel bewertet wird.

Im ersten Kapitel betrachten wir den allgemeinen Zusammenhang zwischen Schlafphysiologie, Schlaf-Wach-Zyklus, die Rolle von Adenosin und den daraus resultierenden Auswirkungen auf kognitive und physiologische Funktionen nach Koffeinkonsum. Der allgegenwärtige Konsum von Koffein, vorwiegend durch Kaffee, ist historisch mit der Reduzierung von Schläfrigkeit und der Steigerung von Vigilanz verbunden. Die dargelegte Arbeit untersucht die variablen Reaktionen auf Koffein, beeinflusst durch unterschiedliche Formulierungen, Dosierungen und genetische Polymorphismen in den Adenosinrezeptoren und Koffein abbauenden Enzymen. Wir werden einen historischen und wissenschaftlichen Überblick über die Schlafforschung angehen, zeitgenössische EEG-Methodologien und ihre entscheidende Rolle bei der Aufklärung der zerebralen Funktion während des Schlafs betrachten. Die Bedeutung des Schlafs von kognitiven Aspekten wie Gedächtniskonsolidierung bis hin zu Funktionen wie neuronale Reparaturen, Beseitigung von Metaboliten und die Wiederherstellungsprozesse werden diskutiert. Wir werden eine Einführung in die verschiedenen Schlafstadien geben, die jeweils durch unterschiedliche EEG-Muster und spezifische physiologische Funktionen charakterisiert sind, um den Grundstein für die Erforschung der komplexen Wechselbeziehungen zwischen Schlaf, Koffeinkonsum und deren vielfältigen kognitiven und physiologischen Auswirkungen zu legen.

Im zweiten Kapitel betrachten wir die Wirksamkeit von Kaffee bei der Minderung der kognitiven Leistungsfähigkeit in Verbindung mit Schlafrestriktion bei Personen mit genetischer Prädisposition für Koffeinempfindlichkeit. In einer Doppelblindstudie wurde die kognitive Leistung von Teilnehmenden, die regulären und entkoffeinierten Kaffee über fünf Nächte mit eingeschränktem Schlaf konsumierten, verglichen. Die Ergebnisse zeigten, dass beide Gruppen erhöhte subjektive Schläfrigkeit berichteten, die Gruppe, die regulären Kaffee konsumierte, jedoch signifikant bessere Leistungen bei Aufgaben zur Aufmerksamkeit und exekutiven Kontrolle zeigte. Wichtig ist, dass diese vorteilhaften Auswirkungen nach einer Erholungsnacht verschwanden, was auf die Vorübergehende Wirksamkeit von Koffein hinweist.

Im dritten Kapitel tauchen wir tiefer in die physiologischen Aspekte des Koffeinkonsums ein und konzentrieren uns speziell auf dessen Auswirkungen auf EEG, Herzfrequenz und autonome Aktivität während des Schlafs. Wir haben Blutplasma in regelmäßigen Abständen untersucht, um die Koffeinkonzentration zu quantifizieren, zusammen mit einer standardisierten Polysomnographie. Es wurde festgestellt, dass Koffein dosisabhängig die Delta-Aktivität und die Herzfrequenz während des Non-REM-Schlafs verringerte. Darüber hinaus erhöhte Koffein die hochfrequenten Spektren der Herzfrequenzvariabilität (HR-HRV), was auf eine erhöhte

parasympathische Aktivität hindeutet. Diese Studie etabliert eine quantitative Konzentrations-Wirkungs-Beziehung zwischen Koffein, EEG-Delta-Leistung, Herzfrequenz und autonomer Aktivität.

Im vierten Kapitel untersuchen wir die Wirksamkeit von Koffein bei der Bekämpfung von Schlafträgheit, einem Zustand von Benommenheit und beeinträchtigter Wachsamkeit unmittelbar nach dem Aufwachen. Mit einer innovativen, verzögerten, pulsierenden Koffein-Freisetzungformel zeigte diese Studie signifikante Verbesserungen beim Übergang von Schlaf zu Wachheit bei Schlafrestriktion. Im Vergleich zu einem Placebo beschleunigte diese Koffein-Formulierung die Reaktionszeit bei psychomotorischen Wachsamkeitsaufgaben, verbesserte die positiven und negativen Affekt-Werte, verlängerte die Kortisol-Aufwachreaktion und verzögerte die Schlaflatenz des Nickerchens.

Insgesamt beleuchtet dies die vielfältige Rolle von Koffein bei der Moderation von Schlaf und kognitiver Leistung. Von der Milderung der negativen Auswirkungen von Schlafmangel bis zur Modulation der Herz- und Hirnphysiologie während des Schlafs bietet Koffein eine zeit- und konzentrationsabhängige Lösung, d.h. die Vor- und Nachteile von Koffein hängen stark von seiner Dosierung, dem Zeitpunkt und individuellen physiologischen Faktoren, wie der genetischen Prädisposition für Koffeinempfindlichkeit, ab. Darüber hinaus eröffnet diese Arbeit neue Wege für die Entwicklung spezialisierter Koffein-Formulierungen, die gezielte Linderung von spezifischen schlafbezogenen Problemen wie Schlafträgheit bieten.

Diese Dissertation zielt darauf ab, die komplexe Beziehung zwischen Koffein, Schlaf, kognitiver Leistung, genetische Polymorphismen und physiologische Wechselwirkungen zu beleuchten. Sie liefert überzeugende Evidenz dafür, dass Koffein die kognitiven und physiologischen Beeinträchtigungen durch Schlafmangel und Schlafträgheit mildern kann. Diese Erkenntnisse können von Nutzen sein, um den Einsatz von Koffein zur Steigerung von Leistung und Wohlbefinden in der Gesellschaft zu optimieren, insbesondere unter schlafbeschränkten Bedingungen.

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# 1.1 Introduction

## 1.1.1 Brief overview and scope of the dissertation

Sleep is a fundamental physiological process, evolutionarily conserved across all mammalian species, thought to be one of the most important physiological processes supporting both brain and body health, as well as mental wellness (Tobler, 2005; Yamazaki et al., 2020). The shift towards a continuously operational society driven by human activities significantly influences both the duration and quality of sleep attainable, introducing widespread sleep restriction or deprivation (Bixler, 2009).

During prolonged wakefulness adenosine levels accumulate in the brain, which signals the need for sleep. As adenosine builds up, it promotes sleepiness and eventually deep sleep, which in turn clears adenosine from the brain and reduces sleep pressure (Reichert et al., 2022b).

Sleep deprivation or restriction not only cause cognitive deficits but are also associated with an extensive range of health issues including, but not limited to, cardiovascular, renal diseases, hypertension, diabetes, cerebrovascular incidents, obesity, and psychological disorders (Colten et al., 2006; Knutson et al., 2007).

The pervasive consumption of caffeine, predominantly through coffee, historically counters increased sleepiness, enhancing attention and vigilance significantly within the context of modern lifestyle-induced chronic sleep reduction (Beaumont et al., 2001).

The response to caffeine is moderated by various factors, including caffeine formulations, dosing, genetic variances in adenosine receptor sensitivity and metabolic enzyme activity, underscoring the necessity for further detailed examination. Furthermore, caffeine's impact extends beyond cognitive performance. Its role in modulating physiological functions during sleep, particularly as observed through alterations in EEG delta power and cardiac autonomic activity, indicates a broader influence that requires comprehensive exploration. These physiological markers not only provide insights into the depth and quality of sleep but also relate to overall cardiovascular health (St-Onge et al., 2016).

The dissertation addresses some of these interactions by exploring how 'real world' coffee consumption affects individuals with chronic sleep restriction and caffeine sensitive via genetic polymorphism. It examines the potential of caffeine to counteract sleep-related cognitive impairments and later investigates its physiological and dose-dependent effects during sleep, specifically on the cardiovascular level and slow wave activity.

Moreover, conventional caffeine formulations may not effectively combat sleep inertia, a particularly troublesome state of impaired vigilance immediately upon waking. Thus, providing potential for developing specialized caffeine formulations that can target specific sleep-related issues without causing unwanted side-effects.

## 1.2 The importance of sleep

### 1.2.1 What is sleep?

Sleep is an inherently reversible condition, characterized by a pronounced decrease in both the awareness of and reactivity to our surroundings (Anafi et al., 2019). This phenomenon was already examined by Aristotle around 350 BC. In his thoughts about the nature of sleep, Aristotle suggested that sleep is an intrinsic aspect of any creature with the capacity for sensory perception. He argued that sleep is essentially the absence of wakefulness, with both states being mutually exclusive and unable to coexist simultaneously. Yet, despite being opposites, he believed they are experienced by the same being, analogously to how health and sickness or strength and weakness can alternate within an individual.

Aristotle assumed that the act of sense-perception involves the soul's movement through the body, localized within a particular organ, and that this process can only occur while awake. He theorized that if this sensory organ were to persist in perception beyond a certain threshold of continuous operation, it would become exhausted and incapable of further activity. Consequently, he deduced that in every living creature, periods of wakefulness must inevitably be followed by sleep to restore this capacity, and vice versa (*On Sleep and Sleeplessness by Aristotle* Translated by J. I. Beare).

Centuries later, numerous hypotheses have been put forth to elucidate the essence of sleep. It was not until technological advancements enabled the scientific study of brain activity that a more empirical approach to understanding sleep emerged. The earliest known measurements of electrical brain activity were conducted by Richard Caton in 1875, who recorded electrical currents from the exposed cerebral hemispheres of rabbits and monkeys.

The turning point in sleep research came with the invention of the electroencephalogram (EEG) by Hans Berger in 1924. This groundbreaking, non-invasive method allowed for the observation of the human brain's electrical activity. Berger noted that brainwave patterns transitioned from rhythmic fluctuations to a more irregular and diminished form upon the closing and opening of the eyes, respectively (Berger, 1929).

Despite this significant discovery, Berger's work was initially met with skepticism by the scientific community. Critics speculated that the EEG readings might be influenced by cardiac or muscle movements, and some even questioned whether the brain's activity could become less rhythmic upon eye opening. Others expressed doubts that the slow rhythmic changes Berger observed could accurately represent neural changes. The validity of the EEG as a trustworthy tool for recording the brain's electrical fields was finally established in 1934 when two British physiologists successfully replicated Berger's experiments, leading to widespread acceptance and recognition of his findings within the scientific domain (Borbély, 2004).

### 1.2.2 How do we measure brain activity?

Electroencephalography (EEG) offers a window into the electrical activity of our most complex organ. To understand what the EEG measures, we need to begin recognizing its immediate potential sources - the potential generators - and the anatomical structures that underly the rhythm and various potential patterns observed in cortical voltage fluctuations.

At the heart of EEG's potential generators lies the cerebral cortex, where the physiological underpinnings are well-established. However, one must distinguish these from the variable potential patterns emerging from the complex interplay of neuronal structures in both cortical and subcortical regions. Since the pioneering investigations of bioelectrical activity by Caton, it has been evident that the potential fluctuations derived from the skull or brain surface originate in the ganglion cells of the central nervous system. Nonetheless, these early insights were limited in explaining the often sluggish potential fluctuations characteristic of the EEG.

However, the advent of intracortical microrecordings, allowed to see that a neuron's action potential, despite its dramatic voltage fluctuation of 80-100 mV, diminishes significantly at a mere distance of 1  $\mu\text{m}$  (Zschocke & Hansen, 2012).

Given its short duration of 1-2 ms, the action potential's contribution to the summations in spontaneous activity remains minimal. Even when recording directly from the cortical surface, individual ganglion cells' action potentials elude detection. However, since every bioelectrical discharge of the brain is ultimately caused by nerve cells as the only active elements of the central nervous tissue, the immediate potential sources of the EEG must have other sources in the cerebral cortex.

The synapses emerge as additional voltage sources in the central nervous system, a revelation made possible by microelectrodes in the cerebral cortex. Activated synapses induce localized membrane potential fluctuations in the postsynaptic neurons. These postsynaptic potentials, unlike the brief action potentials of ganglion cells, develop more slowly and do not adhere to the all-or-nothing law. They tend to overlap in various ways and form slowly changing summation potentials, particularly in dendrites. These summation potentials then become the precursors to the spatially widespread potential fields captured in EEG recordings.

Synapses, varying in their morphology, can be either axosomatic or axodendritic, based on their contact points on the neuron. They also differ in function, being either excitatory or inhibitory. The EEG technique leverages these synaptic activities to measure brain function. By placing electrodes on the scalp, EEG captures the electrical signals from different brain regions, which are then amplified and graphically depicted, showcasing the brain's wave patterns.

While the direct recordings from the cortical surface may not reveal the action potentials of individual ganglion cells, the EEG illuminate the summation potentials formed by synapses and stands as a testament to the complex interplay of neuronal structures within the brain.

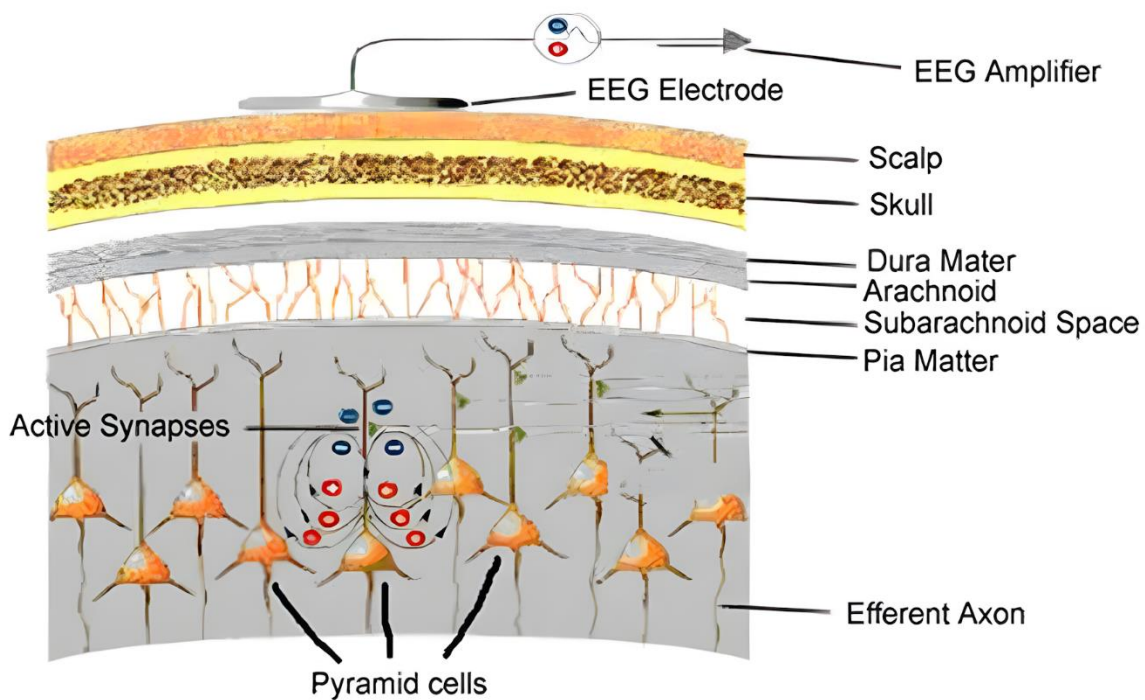


Figure 1. Working principle of Electroencephalography (Figure taken from "EEG & Electrophysiology").

### 1.2.3 The EEG during sleep

The brain is active during sleep, with activity that is specific to each region and network. Traditionally, guidelines are used created by Rechtschaffen and Kales in 1968 to visually score overnight PSG recordings of the electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and electrocardiogram (ECG) (Rechtschaffen & Kales, 1968). The recording is initially broken up into 20- or 30-s epochs, which are then divided into Wakefulness (W), Rapid Eye Movement (REM), and Non-REM (NREM) sleep. According to R&K's guidelines NREM sleep is further divided into stages 1, 2, 3, and 4 (also known as S1, S2, S3, and S4). The major revisions of the guidelines proposed by the American Academy of Sleep Medicine (AASM) in 2007 standards combine the NREM stages S3 and S4 into a single deep sleep stage known as N3, which is also known as slow wave sleep (SWS), leaving NREM divided into a total of three stages (Berry et al., 2017). During sleep, a person typically experiences about four to five cycles of non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS), each lasting around 90 to 120 minutes (Borbély & Achermann, 1992).

Stage N1: the EEG moves from alpha waves with a frequency of 8–13 Hz (common in the awake state) to theta waves with a frequency of 4-7 Hz during stage N1. Also, vertex sharp waves and slow eye rolling movements can be observed. Subjects usually spend approximately 2 to 5% of the total sleep time during this stage, which often is referred to as sluggish sleep or somnolence (Ohayon et al., 2004). During N1, the onset of sleep may be associated with sudden twitches,

hypnic jerks, reduced muscle tone and reduced awareness of the outside world. Some individuals may also experience hypnagogic hallucinations.

Stage N2: K-complexes and sleep spindles ranging from 11 to 16 Hz (most commonly 12–14 Hz) are characteristic of this sleep stage. Conscious awareness of the external environment is lost and muscular activity, as measured by EMG, decreases. Adults spend 45–55% of their total sleep at this stage.

Stage N3 (deep or slow-wave sleep): is characterized by the presence of at least 20% delta waves with peak-to-peak amplitudes greater than 75  $\mu\text{V}$  (EEG standards define delta waves as 0 to 4 Hz, but sleep standards in both the original R&K and the new 2007 AASM guidelines have a range of 0.5–2 Hz). Parasomnias like night terrors, nocturnal enuresis, sleepwalking, and somniloquy occur at this stage. Stage N3 with 20–50% delta waves and a stage N4 with greater than 50% delta waves are depicted in earlier illustrations and descriptions but have been combined to stage N3. From here slow-wave activity (SWA) can be computed, which primarily represents the delta wave component of the EEG, typically defined within the frequency range of 0.75 to 4 Hz (Achermann & Borbély, 1990).

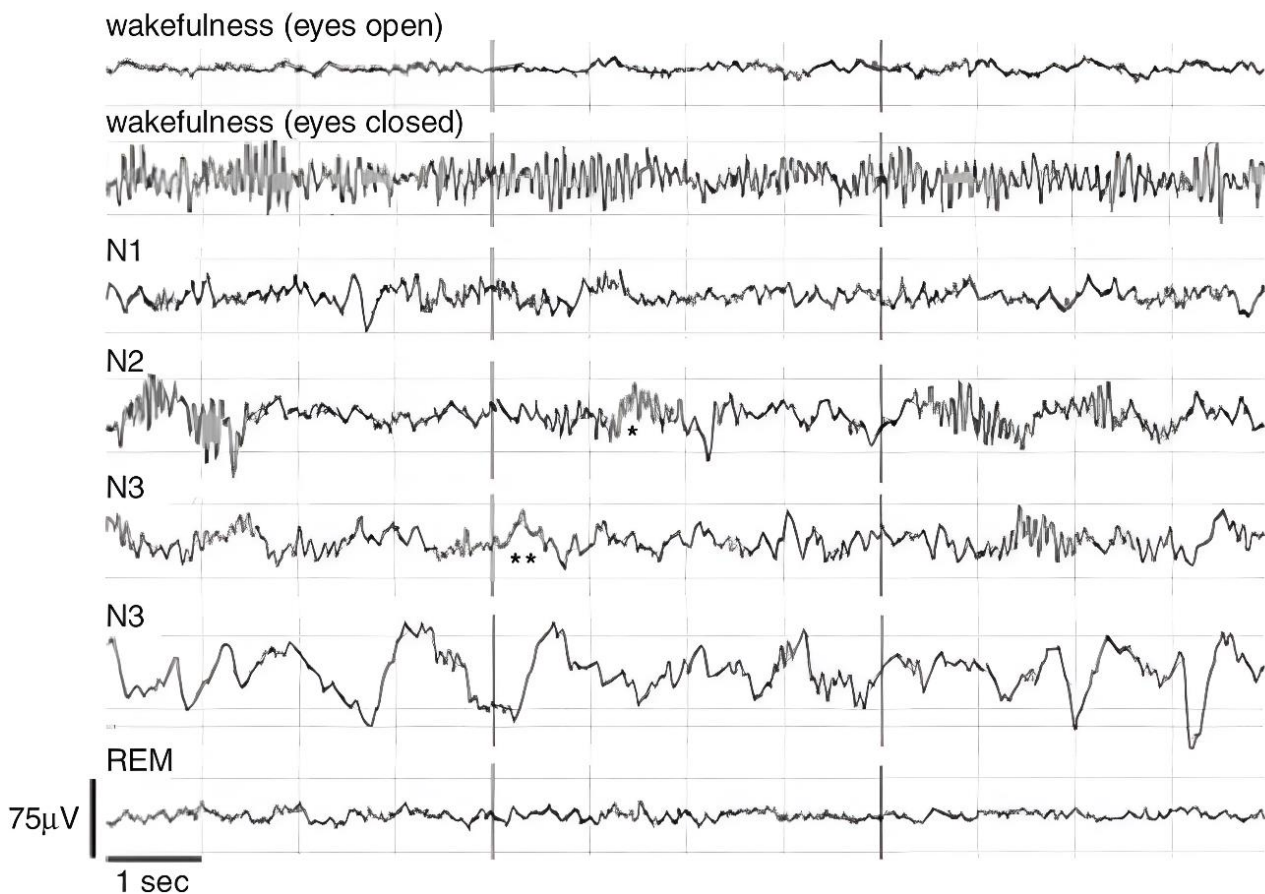


Figure 2. EEG patterns during sleep (from Nir et al., 2013)

REM: The majority of adult humans get 20–25% of their total sleep time in rapid eye movement (REM) sleep (Williams et al., 1966). Rapid low-voltage EEG and rapid eye movements are two of the criteria for REM sleep. This is when most memorable dreams occur. Descending muscular atonia is typical, which also prevents self-harm when physically acting out scenes from vivid dreams at this stage.

#### 1.2.4 Physiological functions and biological imperative of sleep

Despite humans spending roughly one-third of their lives asleep, the precise purpose of sleep remains elusive (Simon et al., 2022). Historically, sleep was considered a characteristic of mammals and birds, identified by specific electroencephalogram (EEG) patterns. However, research from the early 2000s suggests even *Drosophila* exhibit sleep-like states, broadening the scope to non-mammalian and non-avian animals (Hendricks et al., 2000; Shaw et al., 2000). The evolutionary perspective underlines sleep's critical roles in both cognitive processes and housekeeping functions, supporting the theory that sleep is an ancient, conserved aspect of animal biology (Tobler, 2005).

Sleep is integral to cognitive functions, particularly for memory consolidation and synaptic plasticity, supported by complex neurophysiological and molecular frameworks. During non-rapid eye movement (NREM) sleep, hippocampal neurons replay firing sequences associated with waking experiences, a key process for memory consolidation. This has been linked to sharp-wave ripples in the hippocampus, essential for transferring information into short-term memory and enabling information exchange between the hippocampus and cortex, thereby aiding long-term memory consolidation (Davidson et al., 2009; Fujisawa & Buzsáki, 2011; Sejnowski & Destexhe, 2000). Notably, the disruption of these ripples can significantly impair spatial learning and memory (Born & Wilhelm, 2012). The enhancement of synaptic plasticity during sleep, through mechanisms such as synaptic downscaling, allows for the preservation of significant synaptic connections, thus optimizing cognitive performance by improving the signal-to-noise ratio (Tononi & Cirelli, 2014). This process has been described as the Synaptic Homeostasis Hypothesis (SHY), which proposes that during sleep, global synaptic weakening offsets global synaptic strengthening that occurs during wakefulness (Tononi & Cirelli, 2014). The SHY proposes that this renormalization of synaptic weights occurs predominantly during sleep and that it should manifest as a global reduction in synaptic strength. A number of changes in proteins, synaptic efficacy, synapse and dendrite morphology are consistent with predictions of SHY (Chen et al., 2007). This process relies on the regulation of translation initiation and elongation, facilitated by molecular pathways like the mammalian target of rapamycin complex 1 (mTORC1) and translational regulators, which are involved in sustained synaptic plasticity and long-term memory formation (Costa-Mattioli et al., 2009; Santini et al., 2014). Sleep also enables the reactivation of NMDA receptors and CaMKII post-learning, involved in memory consolidation and storage (Wang et al., 2006), while glutamatergic activation in the hippocampus promotes new protein synthesis and cytoskeletal modifications, vital for memory consolidation (Morgado-Bernal, 2011). The regulation of synaptic strength, including bidirectional changes in synaptic transmission and the postsynaptic modulation of AMPA-type glutamate receptors, particularly through the Arc protein, determine lasting synaptic alterations fundamental to learning and memory (Ramirez-Amaya,

2007). The replay firing patterns of hippocampal CA1 ensembles during both quiescent wake and NREM sleep, termed low-probability sequences, occur at speeds significantly faster than those during active spatial experiences, suggesting a compressed consolidation process facilitated by sharp-wave ripples. These ripples, expressed during the depolarized phases of the slow oscillation in both cortex and hippocampus, are important for spatial and temporal coding of information into short-term memory, and the information exchange between the hippocampus and cortex, ultimately reinforcing long-term memory in the cortex (Davidson et al., 2009; Fujisawa & Buzsáki, 2011). However, sleep deprivation can severely inhibit the induction or maintenance of long-term synaptic potentiation (LTP) and depression (LTD), essential mechanisms for learning and memory. Studies in both awake and anesthetized rodents have shown that sleep deprivation impairs hippocampal LTP, with similar detrimental effects observed in conditions of REM sleep deprivation, total sleep deprivation, or sleep restriction, potentially due to alterations in NMDA receptor function, ERK/MAPK activation, dendritic spines, plasticity-related mRNAs or proteins, and concentrations of PDE4 and extracellular adenosine (Campbell et al., 2002; Kopp et al., 2006; McDermott et al., 2003; Romcy-Pereira & Pavlides, 2004).

The glymphatic clearance hypothesis suggests that one of the functions of sleep is to eliminate waste products of metabolism from the brain, such as beta amyloid, soluble proteins, lipids, ions, and small molecules like lactate and adenosine (Porkka-Heiskanen et al., 2002; Xie et al., 2013). The glymphatic system is responsible for the exchange of cerebral spinal fluid (CSF), interstitial fluid (ISF), and blood in the brain. This system is made up of perivascular spaces around penetrating arteries, which are bounded by the end-feet of astrocytes and the endothelium and smooth muscle of the vessel walls (Iliff & Nedergaard, 2013). Water and small molecular solutes enter the astrocytes through aquaporin (Aqp4) channels in the astrocyte end-feet membranes, and then the water and solutes are distributed to the interstitial fluid. The interstitial fluid, along with its waste products, leaves the brain through perivascular spaces around venules and veins draining into cervical lymphatics and venous blood in the dural sinuses (Hladky & Barrand, 2022). During sleep, the perivascular spaces expand significantly (up to 60%) compared to wake, facilitating the flow of ISF through the brain parenchyma and clearance of waste products. The volume of the interstitium and the flow of interstitial fluid are controlled by at least one neuromodulator (e.g. norepinephrine), which is high during wakefulness and low during sleep. The brain depends on glucose and lactate for energy, and astrocytes mediate the delivery of these energy substrates to neurons (Falkowska et al., 2015). The high-energy demands of neural activations during wakefulness call upon the glycogen reserves of the astrocytes, causing increases in lactate levels in the interstitial fluid (Lundgaard et al., 2017).

In the view of sleep's restorative function, emphasizing the interplay between neurochemical and physiological mechanisms, the two-process model proposes that sleep regulation involves the interplay between Process S (sleep homeostasis) and Process C (circadian rhythms) (Borbély & Achermann, 1992). Process S is governed by the accumulation and dissipation of sleep pressure, as evidenced by changes in the EEG slow-wave activity (SWA) within the 0.5-4.5 Hz range. This activity increases with prolonged wakefulness and declines exponentially during sleep, reflecting a feedback mechanism where sleep's intensity and restorative quality are modulated by the duration of prior wakefulness. Conversely, Process C orchestrates sleep timing within the circadian cycle, interacting with Process S to optimize sleep architecture and its restorative

outcomes, highlighting the synergy between homeostatic and circadian mechanisms in sleep regulation. In this framework adenosine receptors mediate the effects of adenosine, which acts as a homeostatic regulator of sleep (Process S). Adenosine's production is correlated to neuronal activity; as neurons fire throughout the day, adenosine accumulates in the extracellular space, leading to an increased drive to sleep, or 'sleep pressure' (Huang et al., 2011). Adenosine and its receptors, particularly A1 and A2A, act as a modulator of the homeostatic drive for sleep through its action on A1 receptors, which facilitate sleep and increase SWA, while A2A receptors aid in the transition from wakefulness to sleep by reducing arousal, effectively acting as sleep gates. Adenosine's significance in sleep regulation is underscored by its complex interactions involving multiple brain regions, cell types, and its dual role in the replenishment of the adenine nucleotide pool (Lazarus, Chen, et al., 2019). This complexity is further elaborated through its impact on sleep-wake cycles and the specific modulation of sleep need and gating by A1 and A2A receptors, respectively (Bjorness & Greene, 2009; Elmenhorst et al., 2007).

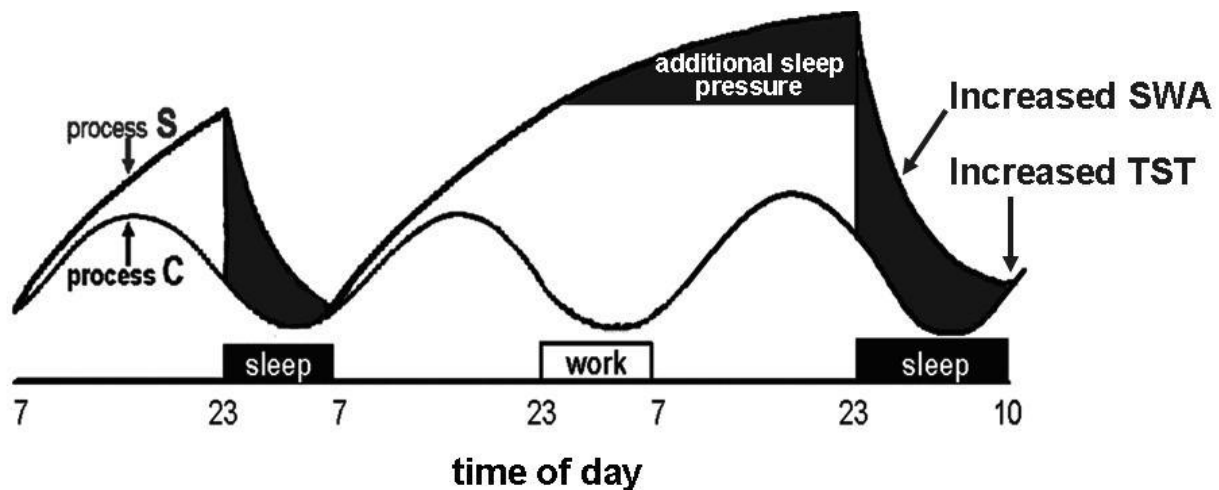


Figure 3. Illustration of the Two-Process Model of Sleep Regulation. Process S is represented by the accumulation of sleep pressure during wakefulness and declining during sleep as pressure is dissipated. Process C follows a circadian pattern, influencing the timing and propensity for sleep (Borbely 1982).

### 1.2.5 Societal implications of sleep deprivation

Sleep deprivation is a growing concern with wide-ranging implications not only for individual health, but also for society at large. The consequences of insufficient sleep are far-reaching, affecting various domains such as public health, economics, and social interactions (Hafner et al., 2017).

Example of tragic accidents linked to insufficient sleep (Coren Stanley, 1996; Ikeda et al., 2019):

- Chernobyl nuclear disaster - In 1986, the Chernobyl nuclear power plant in Ukraine suffered a catastrophic explosion, which released a significant amount of radioactive material into the environment. One of the factors that contributed to the disaster was the fact that some of the workers at the plant were sleep-deprived, which compromised their decision-making abilities.
- Exxon Valdez oil spill - In 1989, the Exxon Valdez oil tanker ran aground on a reef in Alaska, causing a massive oil spill. The captain of the tanker was found to have been sleep-deprived, which impaired his judgment and contributed to the accident.
- American Airlines Flight 1420 - in 1999 a commercial flight from Dallas to Arkansas crashed during landing. The causes of the accident were most likely due to poor weather conditions, crew fatigue since the captain had been awake for more than 16 hours and the first officer for more than 12 hours, combined with poor crew coordination and communication.
- Three Mile Island nuclear accident - In 1979, the Three Mile Island nuclear power plant in Pennsylvania experienced a partial meltdown, which released a small amount of radioactive material. The accident was caused by a combination of equipment failures and human errors, including the fact that some of the operators were sleep-deprived and made mistakes.
- Space Shuttle Challenger disaster - In 1986, the Space Shuttle Challenger exploded shortly after takeoff, killing all seven crew members. One of the contributing factors to the disaster was the fact that some of the engineers who designed the shuttle had been working long hours with insufficient sleep, which compromised their decision-making abilities.
- Deepwater Horizon oil spill - In 2010, the Deepwater Horizon oil rig in the Gulf of Mexico suffered a blowout, causing a massive oil spill. The accident was caused by a combination of equipment failures and human errors, including the fact that some of the workers on the rig had been working long hours with insufficient sleep, which compromised their performance.

These and other incidents have led the CDC and various other health organizations to declare insufficient sleep a public health problem (Hafner et al., 2017). Insufficient sleep impairs vigilance, attention, decision-making, and problem-solving abilities, which is particularly detrimental in precision-critical professions such as healthcare, aviation, and law enforcement, where the consequences can be fatal. Even in roles that are less critical, reduced cognitive function can lead to significant errors, poor judgment, and lower productivity (Liew & Aung, 2021). Moreover,

poor sleep quality not only affects cognitive abilities but also emotional regulation and social interactions. Sleep-deprived individuals are more likely to engage in risky behaviors, suffer mood swings, and show diminished empathy and social understanding (Vandekerckhove & Wang, 2017). Additionally, a lack of adequate sleep is linked to an increased risk of mental health issues, including depression, anxiety, and chronic stress (Sullivan & Ordiah, 2018). The repercussions of sleep deprivation extend to physical health as well, contributing to obesity, diabetes, cardiovascular diseases, and weakened immune function, thereby exacerbating the public health burden (Chattu et al., 2018). The economic impact is also significant, with billions of dollars lost annually due to decreased productivity, increased healthcare costs, and higher accident rates, placing a considerable strain on employers through increased absenteeism, reduced work performance, and elevated turnover rates (Pires et al., 2016). The negative impacts of sleep deprivation also permeate the educational sector. Research shows that poor sleep quality and quantity are associated with decreased learning capacity and academic performance. This is particularly evident in adolescents, where reduced sleep correlates with poorer school performance, cognitive outcomes, and behavioral issues. Delaying school start times has been suggested as an effective measure to combat sleep deprivation in this age group (Hayes & Bainton, 2020; Mitru et al., 2002). Addressing the widespread issue of sleep deprivation requires comprehensive public policy solutions. Workplace interventions, such as educational programs on sleep hygiene, fatigue management, timed napping, increased daytime activity, and modifications to environmental characteristics, have shown to improve sleep quality and reduce sleepiness among employees (Redeker et al., 2019). Programs combining sleep hygiene education and behavioral approaches have been particularly effective for workers with insomnia (Kaku et al., 2012; Nishinoue et al., 2012). However, addressing broader societal factors through public policies, such as regulating work hours and improving access to sleep disorder testing, need further effort in order to alleviate the pervasive effects of poor sleep (Barnes & Drake, 2015).

## 1.3 Adenosine and its role in sleep regulation

Adenosine is a neuromodulator that plays a central role in sleep regulation, linking cellular metabolism to neural activity and ultimately influencing the sleep-wake cycle (Holst & Landolt, 2015; Porkka-Heiskanen et al., 2002). Understanding the biochemistry of adenosine and its relationship to sleep is a prerequisite to comprehend how caffeine affects sleep and wakefulness.

### 1.3.1 Basic biochemistry of adenosine

Adenosine is a purine nucleoside consisting of an adenine ring linked to a ribose sugar molecule (Fig. 5), exerting its physiological effects through adenosine receptors, which are G-protein coupled receptors. Adenosine's diverse physiological effects are attributed to its unique mechanism of formation, and the tendency to redress an imbalance between energy demand and availability, which has even lead to its classification as a "retaliatory metabolite" (Newby, 1984). The synthesis of adenosine begins with the catabolism of adenine nucleotides, which are adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate

(AMP), which are fundamental to cellular energy transactions and numerous cellular functions (Baer & Drummond, 1968; Krebs, 1964). The catabolism of these nucleotides into adenosine is primarily facilitated by the enzymes CD39 and CD73, which dephosphorylate ATP and ADP to AMP and further AMP to adenosine, releasing it into the extracellular space (Mateo et al., 1999; Schulte am Esch et al., 1999). Once released, adenosine interacts with its receptors - A1, A2A, A2B, and A3 - found on various cells including neurons, kidney, heart, hematopoietic precursor cell and immune cells (Borea et al., 2018). The interaction of adenosine with the A1 and A2A receptors subtypes is of particular interest when discussing sleep regulation. Binding to A1 receptors leads to the inhibition of adenylate cyclase via the Gi protein, reducing cyclic AMP (cAMP) levels, thereby reducing protein kinase A (PKA) activity and reducing phosphorylation of various downstream proteins. This typically results in neuronal hyperpolarization and decreased neurotransmitter release, promoting sedative effects (Fredholm et al., 1996).

In contrast, adenosine binding to A2A receptors generally stimulates adenylate cyclase via the Gs protein, increasing cAMP levels, enhancing PKA activity, and modulating neuronal activity implicated in wakefulness and arousal. Furthermore, A2A receptor activation in certain brain regions can stimulate calcium release and increase GABA release, enhancing sleep promotion mechanisms (Basheer et al., 2004). A2AR is also involved in the somnogenic effects of endogenous PGD2 (Huang et al., 2011).

The expression of A1 receptors in the brain mediates sedative and sleep-promoting effects, primarily by inhibiting arousal-promoting neurons, such as those producing hypocretin/orexin in the lateral hypothalamus. This inhibition reduces excitatory synaptic transmission and calcium currents, contributing to the homeostatic regulation of sleep by decreasing the activity of wakefulness-promoting neurons in the basal forebrain and lateral preoptic area (Liu & Gao, 2007; Thakkar et al., 2003). Meanwhile, A2A receptors, particularly concentrated in the striatum and nucleus accumbens, aid in sleep regulation through different pathways by inhibiting dopaminergic wake-promoting circuits, as evidenced in their role in reducing the activity of dopaminergic neurons in wake-promoting regions, such as the ventral periaqueductal gray area (Borea et al., 2018; Zhang et al., 2013).

Finally, while adenosine accumulates in the basal forebrain during wakefulness, increasing sleep propensity, it acts as a negative feedback inhibitor of neuronal activity, particularly in the cholinergic basal forebrain (Porkka-Heiskanen et al., 2002). The breakdown of adenosine is driven by dephosphorylation, deamination, and adenylate-kinase activities (Gupta & Nair, 2006).

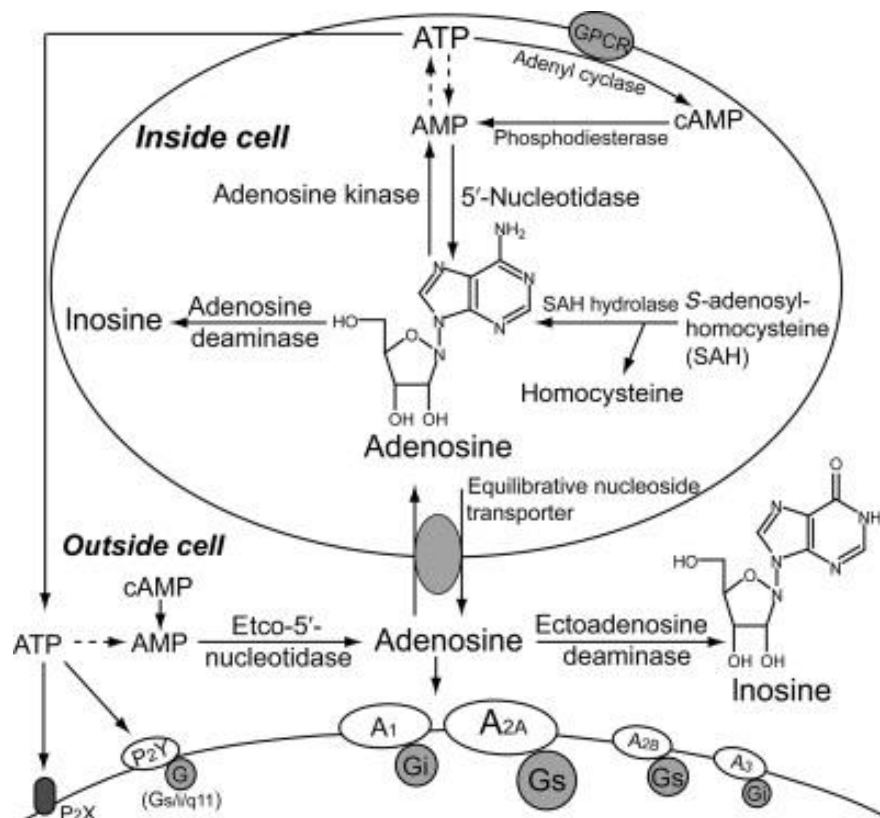


Figure 4. Intracellular and extracellular pathways for the formation and metabolism of adenosine (Huang et al., 2014).

### 1.3.2 The ADORA2A gene

The ADORA2A gene is located on human chromosome 22q11.23. Several single-nucleotide polymorphisms (SNPs) within this gene have been studied for their potential impact on behavior, cognitive functions, and susceptibility to various conditions, including sleep disorders, psychiatric disorders, and response to caffeine (Bodenmann et al., 2012; Oliveira et al., 2019).

ADORA2A polymorphisms refer to variations in the genetic sequence of the ADORA2A gene, which encodes the adenosine A2A receptor. One of the most studied SNPs in the ADORA2A gene is rs5751876 (formally designated as 1976C/T or 1083C/T), often referred to in the context of its impact on caffeine sensitivity, sleep quality, and anxiety (Hohoff et al., 2014). ADORA2A polymorphisms have also been found to affect brain A1AR availability and influence sleep quality, susceptibility to sleep-related disorders and modulation of adenosine receptor availability in anxiety-related brain regions (Hohoff et al., 2020). Individuals with certain variants of this SNP may experience increased anxiety and disrupted sleep patterns in response to caffeine intake (Rétey et al., 2007; Rogers et al., 2010). Another notable SNP is rs5751862, which has been investigated for its association with cognitive performance and vulnerability to sleep deprivation (Erblang et al., 2019).

Variations in the ADORA2A gene can influence the receptor's expression and function, potentially affecting an individual's behavior, cognitive performance, and risk of developing certain conditions. For example, ADORA2A polymorphisms, along with polymorphisms in other genes

like TNF- $\alpha$  and COMT, can create profiles of high vulnerability or high resilience to sleep deprivation, impacting cognitive responses and subjective sleepiness during total sleep deprivation (Erblang et al., 2019).

### 1.3.3 Adenosine receptors and sleep-wake cycle

In the human brain adenosine receptors, are expressed particularly in the striatum, caudate nucleus, putamen, nucleus accumbens, and olfactory tubercle (DeMet & Chicz-DeMet, 2002). Although less pronounced, their presence is also noted in the cortex and hippocampus, and at neuromuscular junctions, affecting presynaptic motor nerve terminals (Baxter et al., 2005; Latini et al., 1996). These receptors are fundamentally involved in regulating the sleep-wake cycle, especially mediating sleep-promoting effects in the subarachnoid space below the rostral forebrain through mechanisms like prostaglandin D2 (PGD2) (Basheer et al., 2004).

It has been shown that sleep deprivation upregulates the A1 adenosine receptor, leading to increased sensitivity to sleep loss (Cohen et al., 2010). This upregulation of the A1 receptor intensifies the impact of sleep deprivation and contributes to the homeostatic regulation of sleep. Following periods of extended wakefulness, recovery sleep restores the availability of A1 receptors (Elmenhorst et al., 2007).

The activation of adenosine A2A receptors in specific brain areas, such as the subarachnoid space of the basal forebrain, significantly increases sleep, underlining its major role in sleep induction. This effect is so profound that adenosine A2A receptors agonists are regarded as among the most potent inducers of sleep, closely mimicking physiological sleep patterns (Hong et al., 2005), while also the main effect of adenosine receptor antagonism, such as via caffeine, are primarily mediated through these types of receptors (Biaggioni et al., 1991; Huang et al., 2014).

In the context of circadian rhythms, besides the action of adenosine receptors, regulation via core clock genes, including transcription factors CLOCK and BMAL1, or NPAS2 and BMAL1 needs to be elucidated. These factors activate the transcription of Period (Per1, Per2) and Cryptochrome (Cry1, Cry2) genes, establishing a feedback loop essential for maintaining the body's internal clock (Franken, 2013). The proteins produced, PER and CRY, form complexes that inhibit their own transcription by acting on the CLOCK/BMAL1 and NPAS2/BMAL1 complexes within the nucleus. This inhibition is temporally relieved by posttranslational modifications, allowing the rhythm to reset.

Sleep homeostasis interacts with this circadian circadian feedback loop, as disruptions in genes like Bmal1, Npas2, Per1, Per2, Cry1, and Cry2 alter delta power during recovery sleep following deprivation (Reichert et al., 2016). Additionally, mRNA levels for clock genes such as Per2 and dbp in the brain change after sleep deprivation, indicating that sleep history can modulate gene expression (Mongrain et al., 2011). Notably, A2A receptor antagonism and knockout of nucleoside transporter 1 reduce the expression of Per2 in the striatum, linking endogenous adenosine levels and dopamine modulation to circadian rhythm disruptions (Ruby et al., 2014).

Per2's expression is influenced by both sleep deprivation and diurnal timing, reflecting its role in sleep homeostasis (Curie et al., 2013). This relationship persists even in mutant mice lacking mPer1 or mPer2, suggesting that sleep homeostasis regulation is dependent of the circadian system (Kopp et al., 2006). Genetic variations in Per2 are associated with alterations in sleep

architecture, particularly affecting slow-wave sleep, and daily rhythms in *Per2* expression are influenced by sleep-wake patterns, increasing during prolonged wakefulness and sleep deprivation (Chang et al., 2016; Hoekstra et al., 2021).

Moreover, circadian changes in membrane excitability and neuronal firing patterns in the suprachiasmatic nucleus (SCN) are driven by fluctuations in  $K^+$  and  $Ca^{2+}$  currents, which peak during the day and diminish at night (Harvey et al., 2020). These changes are modulated by circadian regulation of BK channel activity and linked to clock-controlled intracellular  $Ca^{2+}$  dynamics within SCN neurons. The rhythmic firing patterns in the SCN are supported by various ionic currents, including persistent  $Na^+$  currents and L-type  $Ca^{2+}$  currents, among others, with resting free calcium concentrations in SCN neurons also varying with the circadian cycle, peaking during daylight hours (Colwell, 2000; M. Ikeda, 2004).

## 1.4 Caffeine: a popular adenosine receptor antagonist

Caffeine is one of the most widely consumed psychoactive substances globally, renowned for its stimulating effects (Clark & Landolt, 2017). In the US and Australia caffeine consumption is widespread, with 89-94% of adults consuming it (Fulgoni et al., 2015). The average daily intake is around 186-240 mg, with men generally consuming more than women. While in Switzerland, the mean daily caffeine consumption per adult is 191 mg, with coffee being the main source (Rochat et al., 2019). Coffee is the primary source of caffeine, followed by tea and soft drinks. Consumption increases with age, with a peak in middle age, and is influenced by factors such as gender, race, and occupation (Weinberg & Bealer, 2004).

Caffeine is a natural alkaloid found in various plants, including coffee beans, tea leaves, cacao beans, guarana berries, and yerba maté leaves (Heckman et al., 2010). The widespread occurrence of caffeine in these plants has contributed to the long-standing popularity of caffeine-containing products, particularly beverages (Barone & Roberts, 1984).

### 1.4.1 Chemical structure and properties

Caffeine (Fig. 5) is an alkaloid belonging to the xanthine class of compounds, which is purine base characterized by its three six-membered rings; two of these rings include nitrogen atoms and one incorporates a carbonyl group. This structural arrangement forms the xanthine core, which closely resembles adenosine, allowing caffeine to block adenosine receptors effectively, functioning as an antagonist.

Caffeine is metabolized in the liver to 1,3,8-trimethylallantoin, 1,3,7-trimethylurate, theobromine, theophylline, and paraxanthine (Ferrero & Neims, 1983).

The formation of these metabolites is influenced by factors such as the presence of glutathione, induction of caffeine metabolism, and the activity of cytochrome P-450-dependent enzymes (Grant et al., 1987). The primary metabolite in humans is paraxanthine, and the metabolism of caffeine is mainly mediated by CYP1A2 (de Paula Lima & Farah, 2019; Roberts et al., 1994).

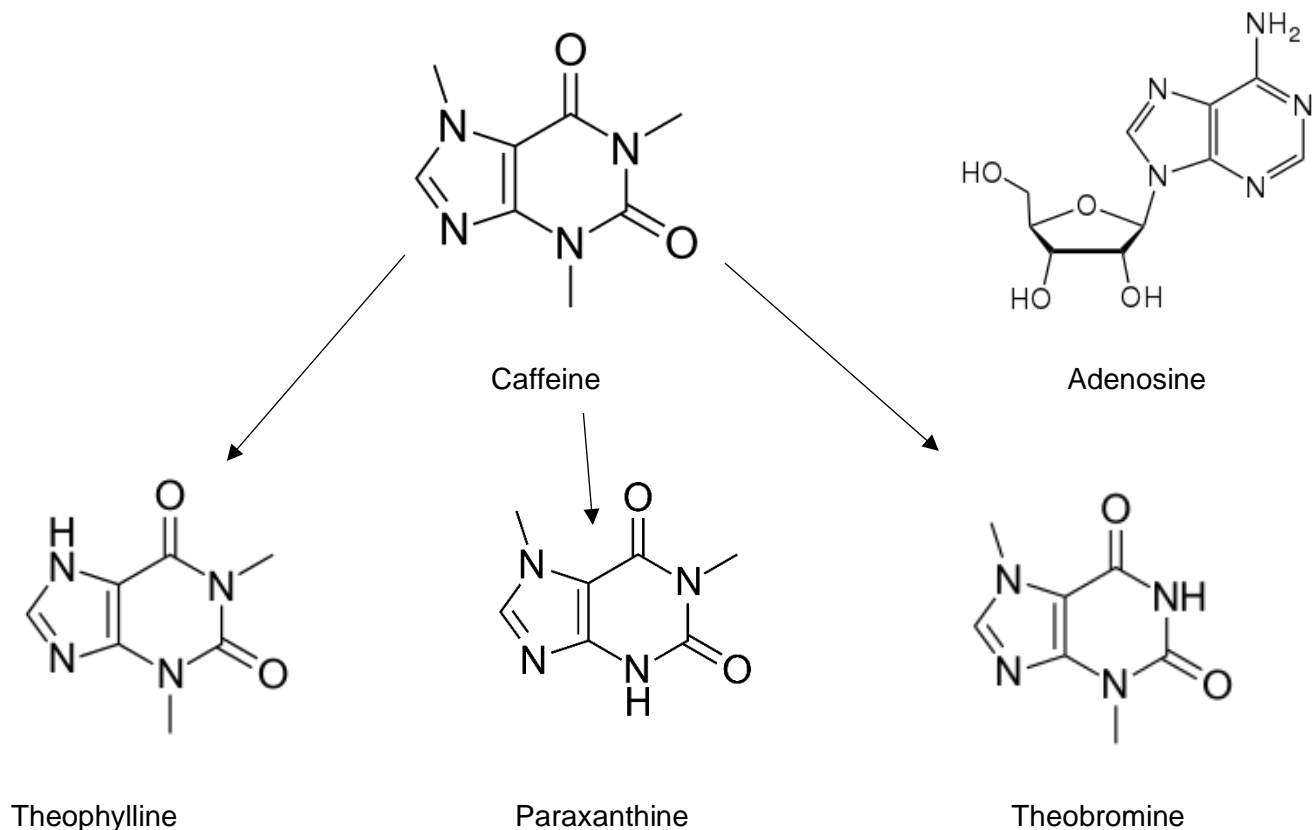


Figure 5 Molecular structure of caffeine, adenosine, theophylline, and theobromine.

Caffeine's interaction with adenosine receptors, specifically its higher affinity for the A2A receptor over the A1, leads to its well-known stimulant effects. The IC<sub>50</sub> (half maximal inhibitory concentration) values for caffeine are around 12  $\mu\text{M}$  for A1 and 2  $\mu\text{M}$  for A2A in brain tissue, reflecting a moderate affinity that is sufficient for noticeable physiological effects at typical dietary intakes (Daly et al., 1983).

Theophylline, a metabolite of caffeine, shows a similar affinity as caffeine for A1 and A2A receptors. Its clinical significance lies in its effect on A2B receptors, which helps in asthma treatment by reducing airway inflammation and relaxing smooth muscles (Makino, 1996). Compared to caffeine, theophylline induces less anxiety and stress, although its potency at A2A receptors is lower than that of caffeine, with IC<sub>50</sub> values of about 10  $\mu\text{M}$  for A1 and 24  $\mu\text{M}$  for A2A (Daly et al., 1983).

Another metabolite, theobromine, exhibits the lowest potency for these receptor types, contributing to chocolate's milder stimulant effects. Despite its structural similarity to caffeine, theobromine does not significantly affect mood or alertness and is thought to influence mainly peripheral physiological changes, rather than the central nervous system effects typical of caffeine (Judelson et al., 2013; Mitchell et al., 2011). Its IC<sub>50</sub> values are higher, around 50  $\mu\text{M}$  for A1 and even lower for A2A, indicating limited efficacy at normal intake levels (Baggott et al., 2013).

Paraxanthine, the main caffeine metabolite, mimics caffeine's thermogenic effects, suggesting a role in energy metabolism (Dulloo et al., 1994).

Caffeine is rapidly absorbed from the gastrointestinal tract, with peak plasma levels reached within 30-120 minutes of ingestion (Heckman et al., 2010). It is extensively metabolized in the liver - up to 99% - producing major metabolites of which 84% is 1,7-dimethylxanthine (Paraxanthine), 12% 3,7-dimethylxanthine (Theobromine) and 4% 1,3-dimethylxanthine (Theophylline). Low doses (70 to 100 mg) of caffeine follow linear pharmacokinetics, whereas higher doses (250 to 500 mg) show reduced clearance and a prolonged elimination half-life, indicating nonlinearity in its metabolism (Bonati et al., 1982).

It is metabolized in the liver by the cytochrome P450 enzyme system, with CYP1A2 predominantly responsible for the major metabolic pathway of caffeine, particularly the N-3 demethylation and oxidation to form urates (Berthou et al., 1991). This enzyme also contributes to the formation of paraxanthine and, to a lesser extent, theobromine and theophylline (Gu et al., 1992). On the other hand, the main responsible for formation of theophylline and theobromine from caffeine is CYP2E1, known as the ethanol-inducible form, indicating that it can influence caffeine metabolism significantly (Gu et al., 1992). However, other P-450 enzymes, including CYP1A1, CYP1A2, CYP2C, and CYP3A4, also play a role in caffeine metabolism (Kot & Daniel, 2008). The rate of caffeine metabolism can be influenced by factors such as cigarette smoking, which can induce P-450A2 activity (Kalow & Tang, 1991; Urry et al., 2016).

## 1.5 The effects of caffeine on sleep and cognition

Caffeine has been shown to prolong sleep latency and reduce sleep efficiency, when consumed in the evening or close to bedtime (Bonnet & Arand, 1995; Landolt, Dijk, et al., 1995).

Additionally, it influences specific aspects of sleep architecture, as decreased slow-wave activity (SWA) and increased power in the higher frequency range of sleep spindles (Carrier et al., 2009; Landolt, Dijk, et al., 1995). Repeated caffeine intake can lead to adaptation in the homeostatic aspect of sleep-wake regulation, as evidenced by increased sleepiness and elevated sleep propensity during caffeine withdrawal (Weibel et al., 2020). Caffeine leads to a dose-dependent increase in wakefulness followed by a prolonged increase in SWA during NREM sleep and when combined with sleep deprivation, caffeine reduces the increase in SWA typically seen after sleep deprivation alone (Schwierin et al., 1996). Chronic caffeine consumption can also increase sleep pressure and alter the sleep-wake cycle (Panagiotou et al., 2019).

The effects on recovery sleep after prolonged wakefulness are less clear, with some studies suggesting minimal disruption and others indicating potential negative repercussions (De Valck et al., 2003; Doty et al., 2017; Paech et al., 2016). The efficacy of caffeine as a countermeasure may depend on the timing and dosage of its administration, with low-dose, repeated administration potentially being more effective (Wyatt et al., 2004).

Epidemiological studies and randomized controlled trials also show that caffeine can prolong sleep latency, reduce total sleep time, and worsen perceived sleep quality (Clark & Landolt, 2017). Nonetheless, it has been shown to have a positive impact on cognition, particularly in terms of alertness, attention, and reaction time after prolonged wakefulness and degraded cognitive performance (Landolt, 2008; van Duinen et al., 2005), which should be weighed against its potential negative effects on subsequent sleep (Snel & Lorist, 2011a).

### 1.5.1 Caffeine as a countermeasure to sleep deprivation.

Caffeine has been extensively studied as a countermeasure to sleep deprivation and sleep restriction, although sometimes with mixed results. It has been shown to improve attention levels in complex cognitive tasks, enhance vigilance, attention, and reaction time across various studies (Alameddine et al., 2014; Jewett et al., 1999; McLellan et al., 2016; Nehlig, 2010; van Vliet et al., 2008). It has also been shown to restore performance on simple psychomotor tasks and objective alertness to mitigate the cognitive deficits associated with sleep inertia or during sleep deprivation (Stepan et al., 2021; Van Dongen et al., 2001; Wesensten et al., 2005).

It may also enhance performance in cognitive performance, problem-solving and reasoning (Jarvis, 1993; Warburton et al., 2001; Zabelina & Silvia, 2020).

However, the effects on learning and memory tasks are less consistent, with some studies reporting facilitatory effects and others reporting no impact (Nehlig, 2010). Similarly, also the effects on higher-order executive function, such as judgment and decision making, are less consistent (McLellan et al., 2016). While it may not significantly improve performance on tasks that require the integration of emotion and cognition (Killgore et al., 2007), it can improve the impact of drowsiness on performance errors (Aidman et al., 2021). Caffeine has been found to improve planning and sequencing abilities, but its withdrawal during sleep deprivation can impair abstract reasoning and concept formation (Killgore et al., 2014).

A review reported that caffeine improved various aspects of cognitive performance such as response time, accuracy on attention tests, executive function, and information processing tasks in sleep-deprived individuals. This highlights caffeine's potential as an effective counter-measure to cognitive impairments associated with sleep loss (Irwin et al., 2020).

Furthermore, studies suggest that caffeine's benefits are more pronounced in preventing performance deterioration rather than enhancing cognitive functions during sleep restriction. Dorrian et al. found that while caffeine did not significantly boost cognitive performance in long-term caffeine-withdrawn individuals, it did prevent further performance decline in those withdrawn overnight (Dorrian et al., 2005). Moreover, Killgore et al. observed that acute caffeine withdrawal during a prolonged sleep deprivation period, adversely affected abstract reasoning and concept formation, indicating that maintaining caffeine consumption during sleep deprivation could prevent some declines in complex cognitive functions (Killgore et al., 2007).

Research involving U.S. Navy SEAL trainees found that moderate doses of caffeine could alleviate many negative effects of sleep deprivation and environmental stress on cognitive functions such as vigilance, reaction time, and memory (Lieberman et al., 2002).

Finally, studies examining the long-term effectiveness of caffeine during sleep restriction have shown that while initial benefits include maintaining alertness, these effects diminish over time (Doty et al., 2017). Habitual caffeine users may require higher doses to sustain vigilance (Dark et al., 2015). Repeated administration of low-dose caffeine, especially during the circadian low point of performance, has been effective in counteracting performance degradation (Hansen et al., 2019; Wyatt et al., 2004). However, individual sensitivity to caffeine can vary significantly, affecting its efficacy (Puckeridge et al., 2011). Additionally, Gardiner et al. noted that caffeine could mildly disrupt recovery sleep following deprivation, potentially affecting sleep maintenance and depth (Gardiner et al., 2023).

A dose-dependent model of caffeine's effects on vigilance has been proposed, with higher doses leading to greater improvements (Ramakrishnan et al., 2014). However, caffeine may lead to overconfidence in performance (Kilpeläinen et al., 2010). Other studies have compared the effects of caffeine with other interventions, such as transcranial direct current stimulation, which was found to be more beneficial in preventing vigilance decrements and improving mood (McIntire et al., 2014). Multiple doses of caffeine have been found to maintain vigilance, attention, and motor coordination during total sleep deprivation (Killgore & Kamimori, 2020).

The effect of caffeine on working memory during sleep restriction or deprivation is unclear.

While some studies suggest that caffeine can offset memory deficits caused by sleep deprivation in mice (Onaolapo et al., 2015), others indicate that it may not significantly improve memory performance (Harrison & Horne, 2000). It may also protect against long-term memory impairment associated with sleep deprivation (Alhaider et al., 2011).

In adolescents, caffeine consumption is associated with reduced total sleep time (Bonnar & Gradisar, 2015). The effect of caffeine on cognitive performance varies with age. One study found that caffeine improved psychomotor and cognitive performance in both young and older adults, with older adults showing a greater improvement (Rees et al., 1999). However, other longitudinal and age-dependent studies reported limited effects of habitual caffeine intake on cognitive decline (van Boxtel et al., 2003; Walters & Lesk, 2016). In older adults high caffeine consumption was associated with better attention and memory performance (Nguyen-Van-Tam & Smith, 2022), while low-dose was reported to have no significant effects on memory and attention in middle-aged and elderly individuals (Schmitt et al., 2003).

Tolerance to caffeine's effects has been observed, but not completely achieved, for example regarding its stimulating effects on mood and wakefulness (Nehlig, 1999).

Individual variability significantly influences the response to caffeine, with factors such as personality traits, age, gender, and smoking behavior impacting caffeine intake (Penolazzi et al., 2012). Genetic variation, particularly in the CYP1A2 or ADORA2A genes, can also affect physiological responses to caffeine, including its ergogenic and anxiogenic effects (Fulton et al., 2018). This genetic variability can further influence cognitive responses to caffeine, with polymorphisms in adenosine and adrenergic receptors playing a role (Renda et al., 2015). The subjective response to caffeine is influenced by factors such as current alcohol use, prior recreational use of stimulants, and baseline arousal levels (Chait, 1992). Genetic polymorphisms in adenosine and adrenergic receptors can also contribute to the variability in blood pressure responses to caffeine (Renda et al., 2012). The metabolism, clearance, and pharmacokinetics of caffeine are affected by various factors, including genetic variability (Nehlig, 2018; Urry et al., 2016).

## 1.6 Sleep and cardiovascular health

Sleep has been linked to cardiovascular health, with various aspects of sleep, including sleep duration, timing, and circadian rhythms, influencing heart health (Kohansieh & Makaryus, 2015; Sum-Ping & Geng, 2022).

Insufficient or excessive sleep duration have been associated with increased cardiovascular mortality (Ahmad & Didia, 2020), while specifically sleep deficiency and deprivation have been linked to cardiovascular disease risk factors such as hypertension, obesity, diabetes, and dyslipidemia (Kohansieh & Makaryus, 2015).

Sleep disturbances, such as sleep apnea, insomnia, and sleep deficiency, have been put into relation with cardiovascular disease through mechanisms such as inflammation, autonomic nervous system disruption, and endothelial dysfunction (Sarode et al., 2023).

Insomnia and sleep loss have been associated with increased cortisol levels, decreased immunity, and increased sympathetic activity, all of which can contribute to the development of diabetes, hypertension, and cardiovascular disease (Khan & Aouad, 2017; Trinder et al., 2012).

Normal and disturbed sleep can be assessed using cardiorespiratory polysomnography, which includes respiratory and circulatory parameters (Podszus, 2009). The importance of sleep in maintaining cardiovascular health is underscored by its restorative nature, particularly REM sleep since it also has been associated with increased risks of cardiovascular diseases, including hypertension and heart failure (Eshera et al., 2024).

### 1.6.1 The heart-sleep connection

The autonomic nervous system modulates multiple cardiovascular parameters during sleep. It is involved in the regulation of heart rate, blood pressure, and vascular resistance, with the parasympathetic and sympathetic arms acting synergistically to maintain homeostasis. This regulation is controlled by the hypothalamic, pontine, and medullary networks (Fink et al., 2018). During non-REM sleep, there is a reduction in blood pressure due to cardiovascular deactivation and baroreflex resetting, while REM sleep is associated with transient increases in blood pressure and baroreflex suppression (Silvani & Dampney, 2013).

The autonomic nervous system also influences the effects of sleep on arterial pressure, with non-REM sleep decreasing arterial pressure through decreased sympathetic vasoconstriction and increased cardiac baroreflex sensitivity (Lo Martire et al., 2018).

However, during REM sleep, there is an increase in arterial pressure due to increased sympathetic activity to the heart and blood vessels (Martire 2017).

Fluctuations during sleep are also observed in heart rate (HR) influenced by various factors, including sleep stage, sleep-related breathing disorders, and circadian rhythm.

Generally, as a person moves from wakefulness into deeper stages of sleep, HR and sympathetic activity progressively decreases with its lowest levels during slow wave sleep. On the other hand, REM sleep is characterized by an increase in HR and considerable fluctuations, which are thought to be under parasympathetic control (ŽEmaitytė et al., 1984).

Sleep-related breathing disorders, such as obstructive sleep apnea, can disrupt the normal balance of the autonomic nervous system during sleep. Despite these disruptions, differences in HR regulation between sleep stages are still present in individuals with sleep apnea (Penzel et

al., 2000). The influence of EEG arousals on HR changes during sleep has also been studied, with a decrease in HR occurring before the arousal and a return to the original value after the arousal. The circadian rhythm of HR and its variability across sleep stages further highlight the complex interplay between sleep and HR regulation (Boudreau et al., 2013).

Poor sleep quality, including sleep deprivation, insomnia, and sleep apnea, is closely associated with an increased risk of cardiovascular diseases and worsened prognosis (St-Onge et al., 2016; Viera et al., 2012). Sleep disturbances can lead to high blood pressure, disrupted circadian rhythm, and exaggerated blood pressure variability. Inflammation, oxidative stress, and antioxidants may mediate the relationship between poor sleep quality and cardiometabolic health (Kanagasabai & Ardern, 2015). Sleep deficiency and deprivation have been linked to cardiovascular risk factors such as hypertension, obesity, diabetes, and dyslipidemia (Kohansieh & Makaryus, 2015). Insomnia and other sleep disorders have also been associated with cardiovascular disease risk (Sherwood et al., 2018).

### 1.6.2 Heart rate variability and its significance

The autonomic nervous system (ANS) significantly influences heart rate variability (HRV) through its dynamic balance of sympathetic and parasympathetic activities (Hejjet & Gál, 2005).

The sympathetic nervous system primarily accelerates heart rate, while the parasympathetic nervous system slows it down. The balance between these two systems modulates the activity of the sinus node pacemaker, influencing the variability observed between consecutive heartbeats (Montano et al., 2009). The proper balance between the sympathetic and parasympathetic nervous systems, both at rest and in response to internal or external stimuli, is important for an organism's flexibility, adaptability, and health (Tonhajzerova et al., 2012). An autonomic imbalance often involves excessive sympathetic activity coupled with reduced parasympathetic activity, as indicated by low HRV. For instance low parasympathetic activity and/or sympathetic overactivity, can lead to tachycardia and is associated with increased risk of cardiovascular adverse outcomes (Vigo et al., 2019). The ANS also modulates HRV through the circadian system, as seen in the clear circadian modulation of cardiovascular variability parameters (Glos et al., 2009). The influence of the ANS on HRV is further supported by the analysis of the influence of the arterial baroreceptor reflex on HRV (Cavalcanti et al., 1996). Despite the complexity of interpreting HRV, it is a valuable marker of ANS activity and can be used to assess and normalize ANS imbalance (Fouradoulas et al., 2019).

The HRV is influenced by various factors including physiological, psychological, environmental, lifestyle, and genetic factors. These factors affect the sympathetic and parasympathetic inputs to the heart, thereby altering HRV (Tiwari et al., 2021). The spectral analysis of cardiovascular signals, particularly the RR period, is a common method used to assess HRV and determine the state of sympathovagal balance under different physiological and pathophysiological conditions (Malliani, 2005). Additionally, HRV can be influenced by central and peripheral feedback mechanisms, which can be quantified through models that consider multiple stressors affecting cardiac activity. The responsiveness of HRV to various stimuli, including physical and affective stressors, reflects the underlying health of the cardiovascular system and can provide prognostic markers in various diseases (Akselrod et al., 1985).

During sleep HRV changes with a shift towards parasympathetic dominance, particularly during the stage of slow wave sleep (Voronin & Biryukova, 2006). This shift is accompanied by a decrease in HRV and total variability, and an increase in low- and high-frequency power. Acute stress can disrupt this pattern, leading to decreased parasympathetic modulation and increased sympathovagal balance during NREM sleep (Hall et al., 2004). Sleep-related disorders, such as obstructive sleep apnea, can further alter the balance of the autonomic nervous system during sleep (Penzel et al., 2000). HRV may also be a useful tool for exploring the sleeping brain, with higher parasympathetic tone during non-REMS and a shift towards sympathetic predominance during REMS (Chouchou & Desseilles, 2014).

## 1.7 Intersecting pathways: caffeine, sleep, and the heart

Caffeine and sleep can have similar effects on cardiovascular health and are interconnected through several physiological mechanisms. Caffeine consumption can lead to poor sleep quality, increase blood pressure, disrupt sleep or reduce sleep duration, which potentially contributes to the development of cardiovascular disease (James & Gregg, 2004; Smith et al., 1993). Sleep disturbances, including sleep deprivation and sleep-disordered breathing, have also been linked to cardiovascular disease (Sum-Ping & Geng, 2022; Wolk et al., 2005).

However, the effects of caffeine on cardiovascular health are generally mild and reversible, with no lasting adverse effects at moderate intake levels (Turnbull et al., 2017). The use of caffeine to mitigate the effects of sleep deprivation on cardiovascular parameters has shown mixed results, with some studies reporting a decrease in heart rate and blood pressure, while others found only modest changes in cardiac autonomic activity (Crooks et al., 2019; Papacocea et al., 2019).

### 1.7.1 Caffeine's effects on heart rate and HRV during sleep

Caffeine has a complex impact on heart rate variability (HRV) and cardiovascular function, which can be influenced by factors such as dosage, time of day, and individual differences in chronotype and caffeine tolerance. Studies have shown that caffeine can increase HRV, particularly in the high-frequency range, suggesting an enhancement of cardiac vagal function (Koenig et al., 2013; Yeragani et al., 2005). This effect tends to be more pronounced in the morning and among individuals with a morning chronotype (Bařda Mastiagina et al., 2005). However, the effects of caffeine on HRV in habitual caffeine consumers are less clear, with some studies reporting no significant differences in HRV parameters (Rauh et al., 2006).

Increased HR, decreased parasympathetic activity and also significant increase in low-frequency/high-frequency ratios during REM sleep have been observed following caffeine consumption (M. Bonnet et al., 2005; Koenig et al., 2013). Yet, some studies report only modest or no changes in cardiac autonomic activity and HRV after caffeine intake (Crooks et al., 2019; Rauh et al., 2006). Furthermore, studies have highlighted a dose-dependent relationship, where higher doses of caffeine can increase HR and sympathetic activity, and decrease parasympathetic activity, potentially leading to adverse cardiovascular events (M. Bonnet et al., 2005). Conversely, in healthy adults, modest amounts of caffeine have been shown to decrease HR and increase

parasympathetic activity, though these effects are generally modest (Crooks et al., 2019; Ketelhut et al., 2022).

Specific studies, such as those involving sleep-deprived medical residents, have indicated that caffeine intake can decrease both HR and blood pressure, altering various cardiovascular parameters (Papacocea et al., 2019). Moreover, the acute effects of caffeine on HRV during exercise have been inconsistent, with some reports of increased HRV before exercise but a decrease during exercise (Yeragani et al., 2005).

Caffeine's impact extends to sleep quality, with intake leading to increased HR, decreased sleep efficiency, and reduced total sleep time, especially when consumed in the evening, potentially leading to poor sleep and further adverse cardiovascular effects (M. Bonnet et al., 2005; E. J. Watson et al., 2016). The relationship between caffeine, heart rate, and sleep quality is complex, indicating significant individual variability and the influence of various factors.

## 1.8 Main research aims of the present thesis

The primary aim of this dissertation is to elucidate the impact of caffeine on cognitive performance during sleep restriction in caffeine sensitive subjects, by examining the concentration dependent effects of caffeine on sleep and cardiac autonomic activity and potential in combating sleep inertia in the morning via a newly developed caffeine release formula. Caffeine is widely consumed for its stimulatory effects, yet the effect size of its impact on physiological and cognitive parameters under various conditions remains underexplored. This research is driven by the necessity to fill some of these gaps.

After a general introduction in this chapter, we will look if "real world" coffee consumption can efficiently attenuate sleep restriction-induced impairments in vigilance and attention in genetically caffeine sensitive individuals. These types of individuals, has been previously been described to be impacted different by caffeine (Rétey et al., 2007).

In chapter 3 we will investigate how different caffeine concentrations impact EEG delta power, heart rate, and high-frequency HRV during sleep. As acute caffeine intake has been shown to impact sleep architecture and autonomic nervous system activity (Crooks et al., 2019; Landolt, Dijk, et al., 1995), this work seeks to establish a more concise concentration-effect relationship.

In chapter 4 we will explore the efficacy of an innovative delayed-release caffeine formulation designed to mitigate sleep inertia and combat impaired vigilance immediately upon awakening. Sleep inertia, characterized by impaired cognitive and physical performance immediately upon waking, poses significant challenges, especially in high-demand environments such as shift work. This chapter evaluates the formulation's impact on psychomotor vigilance, mood, cortisol awakening response, and subsequent sleep propensity, providing assessment of its potential benefits in real-world scenarios.

By dissecting the complex interactions between caffeine dosage, genetic predisposition, and individual physiological responses, these findings aim to optimize caffeine use in mitigating cognitive deficits due to sleep loss.

## 2 Coffee effectively attenuates impaired attention in ADORA2A C/C-allele carriers during chronic sleep restriction

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## 2.1 Abstract

Many people consume coffee to attenuate increased sleepiness and impaired vigilance and attention due to insufficient sleep. We investigated in genetically caffeine sensitive men and women whether 'real world' coffee consumption during a simulated busy work week counteracts disabling consequences of chronically restricted sleep. We subjected homozygous C-allele carriers of ADORA2A (gene encoding adenosine A<sub>2A</sub> receptors) to five nights of only 5 h time-in-bed. We administered regular coffee (n = 12; 200 mg caffeine at breakfast and 100 mg caffeine after lunch) and decaffeinated coffee (n = 14) in double-blind fashion on all days following sleep restriction. At regular intervals four times each day, participants rated their sleepiness and performed the psychomotor vigilance test, the visual search task, and the visuo-spatial and letter n-back tasks. At bedtime, we quantified caffeine and the major caffeine metabolites paraxanthine, theobromine and theophylline in saliva. The two groups did not differ in age, body-mass-index, sex-ratio, chronotype and mood states. Subjective sleepiness increased in both groups across consecutive sleep restriction days and did not differ. By contrast, regular coffee counteracted the impact of repeated sleep loss on sustained and selective attention, as well as executive control when compared to decaffeinated coffee. The coffee also induced initial or transient benefits on different aspects of baseline performance during insufficient sleep. All differences between the groups disappeared after the recovery night and the cessation of coffee administration. The data suggest that 'real world' coffee consumption can efficiently attenuate sleep restriction-induced impairments in vigilance and attention in genetically caffeine sensitive individuals.

## 2.2 Introduction

Undisturbed sleep of sufficient duration is a prerequisite for personal well-being and health and is essential for alertness and cognitive performance necessary for safe and effective functioning. Despite this knowledge, representative national surveys indicate that more than 30% of the adult population in Western societies report sleeping less than the commonly recommended 7–8 h on weekday nights, and roughly 15% regularly sleep less than 6 h (Basner et al., 2014; Tinguely et al., 2014). Consistent with the prevalent co-occurrence of insufficient sleep and excessive daytime sleepiness (Ohayon, 2008), increased sleepiness belongs to the first signs of experimentally induced insufficient sleep (Lo et al., 2012). Similarly, a general slowing in response speed and an increased number of attentional lapses on a psychomotor vigilance test (PVT) is typically observed in normal sleepers when time-in-bed is restricted to 5–6 h over several nights (Balkin et al., 2004; Lo et al., 2012; Van Dongen et al., 2003). These findings corroborate the notion that simple, reaction-time based tasks of vigilance such as the PVT are very sensitive to insufficient sleep (Tkachenko & Dinges, 2018). By contrast, more demanding waking functions such as working memory and response inhibition, which also rely on underlying aspects of attention, appear to be less affected.

A currently prevailing model posits that three separate but interacting attentional networks regulate vigilance (alerting network), orienting (orienting and selection network), and executive attention (executive control network) (Petersen & Posner, 2012). These networks respond in concert to environmental stimuli but are largely independent (Fan et al., 2005). It is assumed that

the alerting network prepares and maintains responses to salient stimuli, the orienting network isolates desired stimuli for processing and directs attention to a target stimulus, and the executive control network allocates attentional resources to manage cognitive workload (Petersen & Posner, 2012).

Sleep deprivation, as well as acute and chronic sleep restriction impair not only vigilance but also executive control, albeit with a lower effect size (Lo et al., 2012). Conversely, tasks assessing orienting appear largely unaffected by acute sleep restriction (Cunningham et al., 2018).

These findings suggest that insufficient sleep differently affects distinct attentional network systems which may provide distinct targets for pharmacological interventions to mitigate sleep-loss induced attentional impairments (Dijk & Landolt, 2019).

To enhance wakefulness in response to sleep restriction, intake of caffeine is highly common, particularly in the morning and early afternoon (Martyn et al., 2018). It is estimated that more than 80% of the world's population consume caffeine on a daily basis, with coffee being the most common dietary source (Clark & Landolt, 2017; Martyn et al., 2018). The average daily caffeine intake per adult equals ~300 mg in Europe and South America, and ~200 mg in the US (Frozi et al., 2018; Heckman et al., 2010; Martyn et al., 2018; Urry et al., 2017). By blocking A<sub>1</sub> and A<sub>2A</sub> receptors of the sleep promoting neuromodulator adenosine, caffeine facilitates cholinergic and monoaminergic neurotransmission in brain regions that regulate vigilance and higher-order attentional processes (Fan et al., 2005). Consistent with this mode of action, acute caffeine administration between ~200–300 mg preserves vigilance and all aspects of attention, in particular when performance degrading factors such as insufficient sleep are present (Jarvis, 1993; Lieberman et al., 2002). Nevertheless, it is currently unclear whether the evidence from studying acute caffeine effects can be translated to real world consumption, where caffeinated beverages are commonly consumed every day. Indeed, it was recently reported that repeated administration of 300-450 mg caffeine per day failed to improve vigilance performance in rested and sleep restricted individuals (Bartrim et al., 2020; Weibel et al., 2020). Remarkably, after a short-lived initial benefit, sleepiness and attentional lapses were even enhanced after caffeine in comparison to placebo when sleep was restricted for more than three nights (Doty et al., 2017). In conclusion, it is currently not known whether daily coffee intake in a dose and timing that mimics 'real world' European habits maintains simple and complex attentional processes during repeated sleep restriction.

Not only dose and frequency of administration, but also pronounced inter-individual differences determine the subjective and objective responses to caffeine and may hamper conclusions on its potency to enhance vigilance and attention. These inter-individual differences are robust and in part genetically determined. More specifically, genetic variants of the adenosine A<sub>2A</sub> receptor gene (ADORA2A), in particular the c.1976T>C variant, were consistently found to modulate neurobehavioral performance during sleep restriction (Bodenmann et al., 2012; Rupp et al., 2013), as well as individual effects of caffeine on self-reported alertness (Rogers et al., 2010), attention network functions (Geiger et al., 2016; Renda et al., 2015) and sleep (Bodenmann et al., 2012; Rétey et al., 2007). These findings suggest that prospective genotyping of the c.1976T>C variant of ADORA2A could provide clearer outcomes on the potential usefulness of coffee as a countermeasure against impaired attention due to insufficient sleep.

To tackle this question, we subjected two carefully matched groups of homozygous C-allele carriers of ADORA2A to repeated sleep restriction and studied the effects of standardized regular

coffee (300 mg caffeine per day) or decaffeinated coffee (< 3 mg caffeine per day) on subjective sleepiness and different facets of attention. We hypothesized that daily coffee consumption in genetically caffeine sensitive individuals attenuates sleepiness and the impairment of performance on all attentional domains during a five-day simulated busy workweek of only 5 h time-in-bed each night.

## 2.3 Materials and methods

All study procedures were approved by the ethics committee of North Rhine (“Arztekammer Nordrhein”), the German Federal Office for Radiation Protection (“Deutsches Bundesamt für Strahlenschutz”) and carried out in accordance with the Declaration of Helsinki. All participants gave written informed consent before participating in the study.

### **Participants**

Prospective study participants aged between 20 and 40 years were recruited through the internal test subject database of the Institute of Aerospace Medicine of the German Aerospace Center (Deutsches Zentrum für Luft- und Raumfahrt e.V.; DLR), as well as advertisements on public websites. Individuals interested in study participation were provided with more details via e-mail and asked to complete a dedicated screening questionnaire. Exclusion criteria included a reported body mass-index (BMI) > 30, presence of sleep-wake disorders and any chronic diseases, habitual nightly sleep duration outside the range of 6-9 h, current shift work and jet-lag, history of head injury, and alcohol or substance abuse. Participants were only evaluated further if they reported no current medication (except contraceptives) and nicotine intake and an estimated habitual caffeine consumption below 450 mg/day. To eligible volunteers, we sent by mail a parcel containing detailed information and a saliva self-collection kit (DNA Genotek Inc., Ottawa, Canada). They were asked to provide a saliva sample for determination of the c.1976T>C polymorphism (SNP-ID: rs5751876) of the gene ADORA2A. A total of 309 OraGene-500 test-kits were genotyped. A detailed flow chart of participant recruitment is provided in Supplementary Fig. S1.

### **Determination of the c.1976T>C genotype**

According to the manufacturer’s instructions, genomic DNA was extracted following an ethanol precipitation protocol using prepIT reagent (prepIT-L2P), such as described previously (Urry et al., 2017). Allele-specific primers were used for selective amplification of each allele (forward primer specific for allele T: 5’ -CGG AGG CCC AAT GGC TAT-3’, forward primer specific for allele C: 5’ -CGG AGG CCC AAT GGCTAC-3’, and reverse primer: 5’ -GTG ACT GGT CAAGCC AAC CA-3’).

Fragments containing 10 ng genomic DNA were amplified using a TaqDNA Polymerase (Thermo Fisher Scientific, Waltham, USA) and a “hot start” procedure. Specifically, an initial denaturing step (10 min, 95 ° C) was followed by 40 cycles of denaturation (1 min, 92 ° C), annealing and elongation (1 min, 60 ° C), using an Applied Biosystems GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, USA). An Applied Biosystems PRISM 7900HT with SDS

software version 2.2 was used for allelic discrimination and fluorescence detection. Consistent with the expected allele frequencies of a Western European study sample (Rétey et al., 2007), 105 homozygous C-allele carriers (34.0%) were identified.

### **Pre-study procedures and experimental protocol**

During at least one week preceding the study, participants were asked to adhere to a regular sleep-wake schedule of 9 h of sleep, starting at 22:00/23:00, and 15 h of wakefulness. They wore a wrist activity monitor and completed a sleep-wake diary, to verify compliance with this instruction. Naps and caffeine, alcohol and medication intake were not permitted (occasional medication intake unknown to interfere with sleep or performance could be allowed upon consultation).

The experimental portion of the study was conducted under controlled conditions in a dedicated, state-of-the-art research facility of the Institute for Aerospace Medicine at the DLR's headquarter in Cologne (<https://www.dlr.de/envihab/>). Upon arrival, prospective study participants were pseudo-randomly assigned to one of two coffee groups, stratified by sex, age and BMI, to either receive standardized regular coffee or decaffeinated coffee during chronic sleep restriction. A member of the medical staff informed all volunteers about the objectives, as well as the risks of the study. Adherence to all pre-study instructions, including urine toxicological screening and saliva caffeine quantification, was verified. In addition, in the first night following arrival at the laboratory the absence of sleep-wake disturbances was confirmed in an adaptation/screening night. Individuals with an apnea-hypopnea index higher than 10 and a periodic leg movement index higher than 15 were excluded from study participation.

The experimental protocol consisted of 9 consecutive days and nights (Fig. 6), aimed at investigating the effects of coffee intake on subjective state, attention, declarative memory, decision making, risk taking, sleep architecture, the sleep and waking EEG, as well as cerebral adenosine A1receptor binding in vivo before, during, and after recovery from sleep restriction. After the adaptation/screening and two baseline nights (BS1 & BS2; either from 23:00–07:00 [n = 14] or from 00:00–08:00 [n = 12], according to each participant's self-selected sleep-wake schedule during the pre-study weeks), sleep was restricted to 5 h time-in-bed during five consecutive days (CS1-CS5; either from 02:00–07:00 [n = 14] or from 03:00–08:00 [n = 12]). The study was concluded with an 8-h recovery night (RN; either from 23:00–07:00 [n = 14] or from 00:00–08:00 [n = 12]). All measurements and recordings were scheduled at identical times awake.

All participants slept in their own single bedroom, where during the day test sessions at the computer took place at regular time intervals. When not engaged in sleep or cognitive testing, participants remained in a common living area to read, eat, play games, or watch television and movies. Light intensity was set at <100 lux during waking hours to minimize light's acute alerting effect and impact on circadian rhythms.

The volunteers were not allowed to leave the research facility during the entire experimental

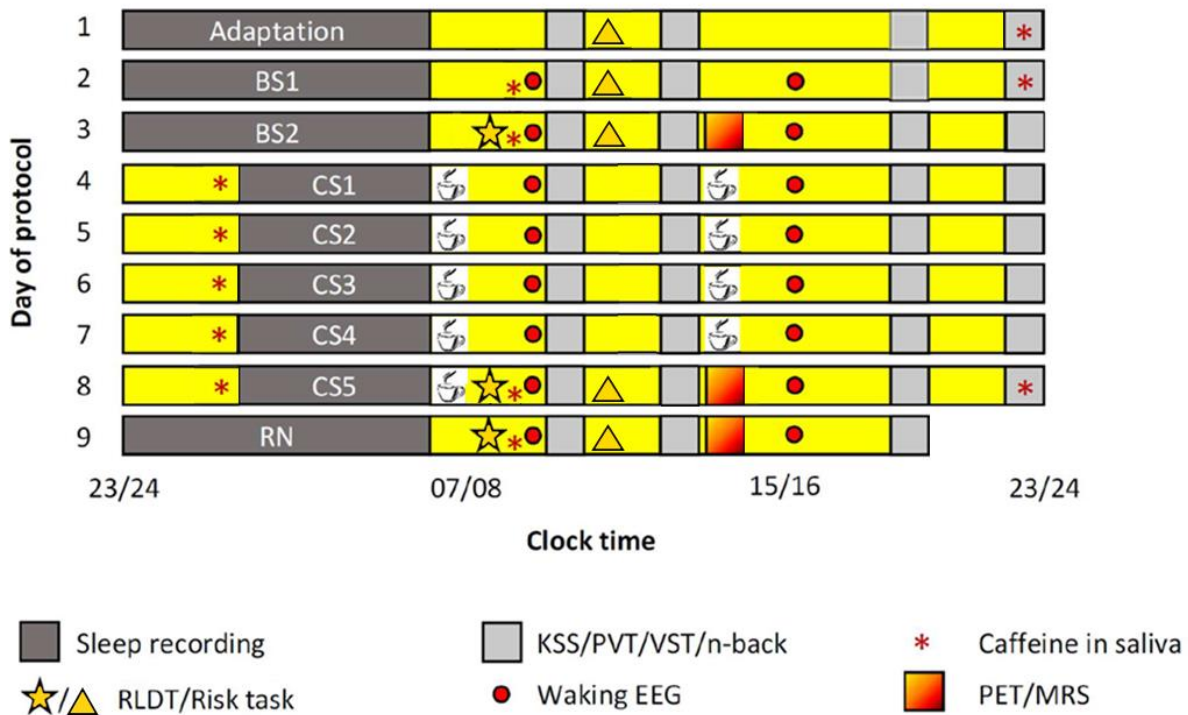


Figure 6: Experimental protocol. Dark grey shading: sleep opportunities recorded with standard polysomnography. Adaptation: adaptation/screening night; BS1, BS2: baseline nights; CS1-5: sleep restriction nights; RN: recovery night. Yellow shading: monitored wakefulness under controlled laboratory conditions. Light grey shading: neurobehavioral test battery consisting of Karolinska Sleepiness Scale (KSS), psychomotor vigilance test (PVT), visual search task (VST), and visuo-spatial and letter n-back tasks. Red asterisks indicate the times of saliva collection for caffeine quantification. Coffee mugs indicate coffee administration. Red circles: high-density waking EEG recordings. Yellow asterisks: reversal learning decision task (RLDT). Yellow triangles: risk task.

period. During the study, naps, smoking, caffeine (except experimental coffee intake), alcohol, medications, and sports were not allowed. Violation of protocol instructions lead to exclusion from further participation in the study. During the pre-study procedures, at screening and in the course of the experiment, 42 individuals needed to be excluded (referred to as “late drop-outs”) (Supplementary Fig. S1). The typical reasons for exclusion included non-compliance to the imposed sleep-wake schedule, positive toxicological screening, and increased apnea-hypopnea or periodic-leg-movement index in the screening night. Twenty-seven homozygous rs5751876 C-allele carriers completed the study. Because one participant of the regular coffee group performed outside the normal range on virtually all cognitive tasks and her data differed by >2 standard deviations from the values of the other participants, this dataset was excluded from the analyses. The demographic characteristics of the remaining 26 individuals are summarized in Table 1. The two experimental groups did not differ on any demographic criteria considered.

## Coffee preparation and administration

Two batches of coffee and high-quality, electric drip filter coffee machines (Tchibo type 5794 and 2855) were obtained from the same manufacturer (Tchibo GmbH, Coffee Technology, Hamburg, Germany).

The coffee was brewed according to detailed instructions provided by the manufacturer, whose pre-study analytics confirmed that adherence to the brewing instructions produced a content of  $101 \pm 0.6$  mg (SD) caffeine per 200 g of regular coffee and  $2.4 \pm 0.05$  mg caffeine per 200 g of decaffeinated coffee (data available on file). On all days following sleep restriction, 400 and 200 g regular (coded as batch “162”) or decaffeinated coffee (coded as batch “833”), respectively, were administered in double-blind fashion at 07:30/08:30 and 14:00/15:00. A daily dose of ~300 mg caffeine roughly matches the estimated habitual caffeine intake patterns from wastewater analyses in Zurich, Switzerland (Gracia-Lor et al., 2017). On sleep restriction day 5, the midday coffee intake was omitted to avoid interference with the scheduled positron emission tomography (PET) scanning (Fig. 6). All members of the research team were blind to the identity of the two coffee batches throughout data collection and statistical analyses of the subjective and behavioral data.

## Subjective sleepiness

To quantify the evolution of subjective sleepiness throughout the experimental protocol, the Karolinska Sleepiness Scale (KSS) was administered before each test session devised to assess the different components of attention (see below). The KSS is a widely validated 9-point Likert scale to estimate the subjects’ self-reported momentary level of drowsiness/sleepiness. It ranges from “1” (extremely alert) to “9” (extremely sleepy, fighting sleep) (Åkerstedt & Gillberg, 1990).

*Table 1: Demographic characteristics of study participants. Values represent means and standard deviations. All study participants were ADORA2A c.1976T>C homozygous C/C allele carriers. MCTQ = Munich Chronotype Questionnaire, values represent time of mid-sleep on free days corrected for sleep debt, ESS = Epworth Sleepiness Scale, PANAS = Positive and Negative Affect Schedule Questionnaire, Body Mass Index and habitual caffeine intake per day were estimated from recruitment survey, Genotype was determined by analyzing DNA from a saliva sample. P-values are derived from a two-tailed, unpaired t-test.*

	Regular coffee	Decaffeinated coffee	p value
n	12	14	
Gender ratio	6 female / 6 male	6 female / 8 male	
Age (years)	$29.9 \pm 5.3$	$28.6 \pm 5.4$	0.42
Chronotype (MCTQ)	$04:25 \pm 56$ m	$04:28 \pm 51$ m	0.82
Epworth Sleepiness Scale	$5.9 \pm 3.1$	$4.9 \pm 2.7$	0.37
PANAS (Positive / Negative)	$35.2 \pm 3.2$ / $13.8 \pm 2.9$	$33.8 \pm 10.6$ / $13.7 \pm 3.2$	0.62 / 0.96
Beck Depression Inventory (BDI-II)	$1.8 \pm 2.4$	$1.4 \pm 1.6$	0.68
Body mass index (kg/m <sup>2</sup> )	$23.2 \pm 3.2$	$23.6 \pm 2.5$	0.85
Habitual caffeine intake (mg /day)	$156 \pm 124$	$113 \pm 91$	0.25

### Testing attention network functions

To study the effects of coffee intake during chronic sleep restriction on different functional aspects of attention, a 35-min test battery was administered on all experimental days at 09:00/10:00, 12:00/13:00, 18:00/19:00, and 21:00/22:00 (Fig. 6). To examine all three attentional networks, each test session included validated versions of a psychomotor vigilance test, a visual search task, and a visuo-spatial and letter (verbal) n-back task.

### Vigilance and alerting network

The psychomotor vigilance test (PVT) is a gold-standard measurement of sustained vigilant attention in sleep and chronobiology research (Dinges & Powell, 1985). This test relies on measuring the reaction time (RT) to a digital ms-counter on a computer screen that starts to scroll randomly ~100 times with variable inter-stimuli intervals between 2 and 10 s over a test duration of 10 min. The RTs below 100 ms were defined as errors of commission and excluded, whereas the RT above 500 ms were defined as errors of omission (lapses) and also omitted from the analyses of mean RT. Speed (1/RT), the number of lapses, accuracy, and the log-transformed signal-to-noise ratio (LSNR) on the PVT were analyzed. Accuracy was defined as the sum count of true positives over the total number of stimuli, thus also taking into account lapses (RT > 500 ms) and false positive responses (RT < 100 ms). The LSNR was previously suggested to quantify PVT performance with high sensitivity, stability, normal distribution, and absence of floor and ceiling effects (Chavali et al., 2017). This novel measure of PVT performance is independent of the absolute position on the metric scale.

The signal-to-noise-ratio (SNR) of PVT performance was approximated with the following formula (Chavali et al., 2017):

$$SNR \approx \frac{N \left( \sum_{i=1}^N w_i S_i \right)^2}{\sum_{i=1}^N \left[ w_i \left( S_i \sum_{i=1}^N w_i - \sum_{i=1}^N w_i S_i \right)^2 \right]} + 1$$

where  $S_i = 1/(RT_i - C)$ ,  $w_i = 1/(r^2 S_i + 1)$ ,  $C = 100$  ms,  $r^2 = 196$  ms,  $RT_i$  is the  $i^{\text{th}}$  RT (in ms), and  $N$  is the number of trials in the PVT session.  $RT < 100$  ms (i.e., false starts) were not included. The log-transformed form of this metric as  $LSNR = 10 \log_{10} (SNR)$  expressed in decibel (dB) was analyzed.

### Orienting and selection network

The visual search task probes the distinct subsystems of the attention network, which relate to searching and selecting stimuli for further processing (orienting) (Pashler, 1987). In the visual search paradigm employed here, the study participants were instructed to find a target item as fast and precisely as possible (i.e., the digit '2') on a display cluttered with distractor items (i.e., the digit '5') (Santhi et al., 2007).

The target was either present or not present and the set size of search items varied between 10, 20, 30 and 40. Speed (excluding RTs < 100 ms) and accuracy (i.e., sum of true positives and true negatives divided by total amount of responses) were analyzed.

### **Executive control network**

A visuo-spatial and a letter n-back task were employed to probe working memory and the executive control network responsible for the allocation of attentional resources (Owen et al., 2005). With increasing cognitive workload, these tasks require the short-term managing and updating of information and as such tap into the executive control of attention. Participants were presented with a series of dot positions and letters on a computer screen and asked to press a button if the current stimulus was presented 1, 2, or 3 steps back. Each of the 3 cognitive workload levels consisted of 20 targets and 40 non-targets. The data were analyzed for speed (including only correct answers and RTs > 100 ms for calculating average values) and accuracy of task responses. Accuracy was defined as the sum of hits and correct rejections, divided by the amount of total responses.

### **Quantification of caffeine and caffeine metabolites in saliva**

Saliva samples for the quantification of caffeine and its main metabolites, paraxanthine, theobromine, and theophylline were collected in Salivette® tubes (Sarstedt, Germany) on all experimental days at bedtime (except for RN where no coffee was administered), as well as in the morning of experimental days B1, B2, CS5 and RN (Fig. 6). The samples were stored at -20 °C and only quantified when the analyses of the subjective and behavioral data were completed. For metabolite quantification, internal standards (IS) had to be prepared consisting of caffeine, theophylline, theobromine and caffeine- <sup>13</sup>C<sub>3</sub>, purchased from Sigma-Aldrich (St. Louis, USA), and paraxanthine purchased from Cerilliant (Texas, USA). All chemicals used were of the highest grade available. For sample preparation, 280 µl of saliva, 70 µl of the IS (8 µM caffeine- <sup>13</sup>C<sub>3</sub>) and 1000 µl of ethyl acetate were added to a tube. Samples were shaken for 10 min and centrifuged (5 min, 10'000 rpm). 800 µl of the supernatant was transferred into an auto-sampler vial and evaporated to dryness under a gentle stream of nitrogen and reconstituted in 250 µl of an eluent-mixture (95:5, v/v). Calibrator (Cal) and quality control (QC) samples were prepared with the same sample preparation, but 70 µl of the Cal or QC solutions were added before adding 930 µl of ethyl acetate. The saliva samples were analyzed using an ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher, San Jose, CA), coupled to a linear ion trap quadrupole mass spectrometer 5500 (Sciex, Darmstadt, Germany). The mobile phases of the UHPLC consisted of water (eluent A) and a mixture (70:30 v/v) of methanol and acetonitrile (eluent B), both containing 0.1% of formic acid (v/v). The flow rate was set to 0.45 ml/min with the following gradient: start conditions 95% of eluent A, decreasing in 3 min to 80%, and a quick decrease to 2% A within 0.5 min, holding these conditions for 1 min and then switch to the starting conditions for a 1 min re-equilibration. Injection volume was 5 µl. A Kinetex Biphenyl column (50 × 2.1 mm, 1.7 µm) (Phenomenex, Aschaffenburg, Germany) was used for the separation of the analytes. Mass spectrometer (MS) was operated in positive electrospray ionization mode with scheduled multiple reaction monitoring (MRM) (Kondrat et al., 1978) with a detection window of 35 s and a target scan time of 1.1 s. Three MRM transitions were used for each analyte. For

analyte quantification, peak areas were integrated and divided by the peak area of the IS. Calibration samples were fitted with a least-squares fit and weighted by  $1/x$ .

The caffeine and metabolite concentrations in the final five study participants (two members of the regular coffee group and three members of the decaffeinated coffee group) could not be analyzed because the UHPLC/MS system was not available. The results of the pharmacokinetic analyses thus rely on ten volunteers who received regular coffee and eleven volunteers who received decaffeinated coffee.

### **Data analyses**

In this manuscript, we report the effects of common coffee intake on subjective sleepiness and the distinct components of attention (vigilance, orienting and executive control) during chronic sleep restriction.

All analyses were conducted using R version 4.0.0 (R Core Team, 2018) and RStudio Version 1.2.5042 (RStudio, Inc.). Data were analyzed via linear mixed effects models (R package lme4 v.1.1.23 and lmerTest v.3.1.2) using residual maximum likelihood estimates to fit the model and maximum likelihood for omnibus analysis of variance (ANOVA). Factors included 'day' (BL [mean of BS1 and BS2], CS1, CS2, CS3, CS4, CS5, RN), 'group' (coffee batch '162', '833'), cognitive 'workload' (1-, 2-, 3-back) and their interactions as fixed effects, whereas 'study participant' and 'set size of search items' (visual search task) were added as random effects when appropriate. Distribution of residuals and goodness of fit was checked in all models and compared to the results of quantile and robust regression methods (R package MASS v.7.3.51.6). In all Figures, group means and 95% confidence intervals are presented, based on 1000 bootstrap replicates (Efron & Tibshirani, 1993). Post-hoc general linear hypothesis tests were computed to compare groups on each day of the study, when 'day' x 'group' interaction terms were significant. To correct for multiple comparison, the Benjamini-Hochberg procedure was applied (R package multcomp v. 1.4.13). To quantify the effect size, Cohen's  $d$  measures were computed for each day of the study (J. Cohen, 1988).

All results of the linear mixed effects model ANOVAs are summarized in Supplementary Tables S1 (self-rated sleepiness and PVT), S2 (visual search task), S3 (visuo-spatial and letter n-back tasks), and S4 (caffeine and metabolites). The statistical analyses testing differences from baseline are illustrated in Supplementary Tables S5-S8.

## 2.4 Results

The baseline assessments of self-rated sleepiness did not differ between the two groups. By contrast, cognitive performance was not uniformly distributed across all subjects. To avoid overestimating the effects of sleep restriction and coffee intake, individual performance measures were linearly centered to the mean baseline value of all participants (referred to as "normalized to baseline"). Moreover, deviations across subjects, were modeled during statistics as random effect. The behavioral data were averaged on each study day and the deviations from the normalized baseline across the experimental protocol are illustrated.

### **Subjective sleepiness**

Self-rated sleepiness scores increased after sleep restriction day 2 in both groups, irrespectively of whether participants received regular coffee or decaffeinated coffee ('day':  $F_{6,672} = 17.50$ ,  $p < 0.001$ ) (Supplementary Fig. S2; Supplementary Table S1). Although the mean KSS rating was slightly attenuated on sleep restriction day 1 after regular coffee administration and appeared to rise less steeply in the regular coffee group when compared to the decaffeinated coffee group ('day' x 'group' interaction:  $F_{6,672} = 3.64$ ,  $p = 0.001$ ), no significant difference between the groups was detected on any day of the experimental protocol. Interestingly, self-rated sleepiness remained elevated after the recovery night in the regular coffee group, but not in the decaffeinated coffee group (Fig. S2).

### **Psychomotor vigilance test**

Speed, lapses and accuracy on the PVT deteriorated with increasing sleep restriction, yet the impairment was attenuated in the regular coffee group when compared to the decaffeinated coffee group ('day' x 'group' interactions: speed:  $F_{6,672} = 7.72$ ; lapses:  $F_{6,672} = 3.69$ ; accuracy:  $F_{6,672} = 4.52$ ;  $p_{\text{all}} < 0.001$ ) (Supplementary Table S1). When compared to baseline, the performance impairment in the decaffeinated coffee group started between sleep restriction day 1 (speed) and 3 (lapses and accuracy) and persisted until the day after the recovery night (Fig. 7). By contrast, in the group receiving regular coffee, PVT speed was faster than in baseline on sleep restriction days 1 and 2 but fell below baseline on sleep restriction days 4 and 5. The slowing in response speed persisted after recovery sleep. A moderate performance impairment operationalized as an increased number of lapses and reduced accuracy on the PVT was evident on sleep restriction day 5.

The differences between the two groups were not uniform throughout the protocol. Mean speed was faster in the regular coffee group than in the decaffeinated coffee group on day 1 through 3 of chronic sleep restriction, while we observed no differences in PVT speed on sleep restriction days 4 and 5. By contrast, on sleep restriction days 3 and 4, the regular coffee group produced less attentional lapses and performed more accurately than the decaffeinated coffee group. More specifically, simultaneously correcting for errors of commission and errors of omission, accuracy remained stable in both groups on sleep restriction days 1 and 2. Afterwards, it steeply decreased with accumulating sleep loss in the decaffeinated coffee group, whereas this decrease was delayed and attenuated in the regular coffee group. The benefit of regular coffee intake was no longer statistically significant on sleep restriction day 5 (Fig. 7; Supplementary Table S5).

To further characterize the performance differences between the groups, the fidelity of information processing in cognition was operationalized as LSNR, a recently proposed novel measure of PVT performance (Chavali et al., 2017). The LSNR was increased in the regular coffee group when compared to baseline on sleep restriction days 1 through 4 and higher than in the decaffeinated coffee group on restriction days 1 through 3 ('day' x 'group' interactions:  $F_{6,672} = 9.54$ ,  $p < 0.001$ ; (Supplementary Table S5). Cognitive information processing fidelity slightly increased in both groups from baseline to sleep restriction day 1, was highest in the regular coffee group on day 2, and decreased in both groups thereafter ('day':  $F_{6,672} = 7.99$ ,  $p < 0.001$ ).

All group differences on the PVT showed large effect sizes ( $d$  all  $> 0.8$ ). No differences in any variable were present following the recovery night (Fig. 7).

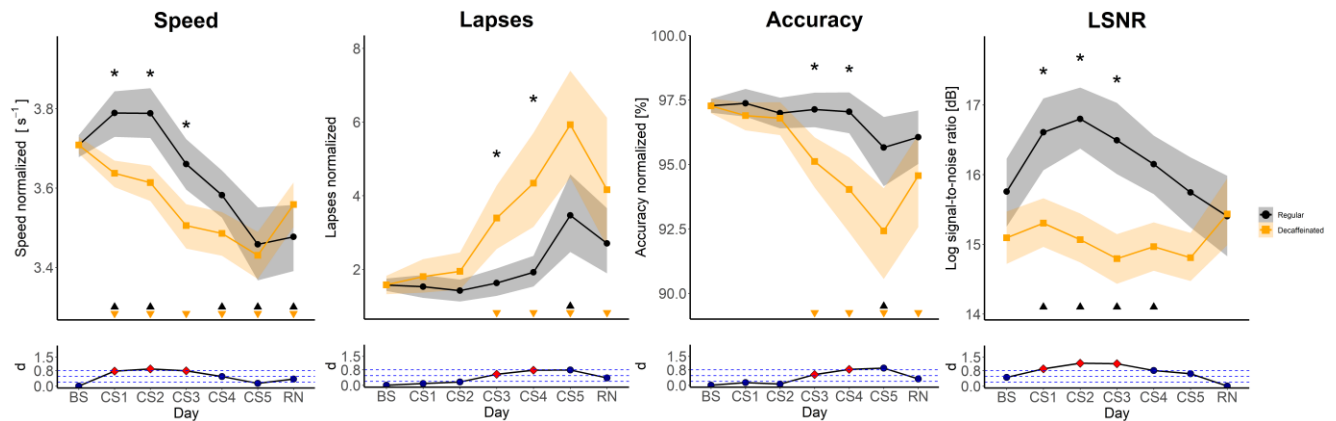


Figure 7: Evolution of vigilance across the experimental protocol. On each study day, performance on four psychomotor vigilance test (PVT) sessions spread over the entire day were averaged. Lines and shaded areas represent means  $\pm$ 95% confidence intervals in the groups receiving either regular (black;  $n = 12$ ) or decaffeinated coffee (orange;  $n = 14$ ). Black (regular coffee group) and orange triangles (decaffeinated coffee group) above the x-axis represent Benjamini-Hochberg corrected differences ( $p_{corr} < 0.05$ ) from baseline (Supplementary Table S5). Stars indicate significant differences between the groups:  $*p < 0.05$ . The corresponding effect sizes (Cohen's  $d$ ) are represented underneath. Dashed blue lines separate regions of "small", "medium" or "large" effect size. Red dots indicate a significant group difference ( $p < 0.05$ ).

### Visual search task

Mean response speed on the visual search task was dependent on the set size of visual distractors (not shown) and the presence of the visual target. Both, when the target was present and when it was absent, response speed remained stable throughout the experiment in the regular coffee group, while response speed in the decaffeinated coffee group was slower than in baseline on virtually all days of sleep restriction and after the recovery night (Fig. 8). The regular coffee group performed moderately faster than the decaffeinated coffee group on sleep restriction day 5 when the target was present ('day' x 'group' interaction:  $F_{6,2759} = 3.08$ ,  $p = 0.005$ , 'group':  $F_{1,26} = 3.28$ ,  $p = 0.082$ ) and on days 4 and 5 of sleep restriction when the target was absent ('day' x 'group' interaction:  $F_{6,2759} = 4.83$ ,  $p < 0.001$ , 'group':  $F_{1,26} = 4.29$ ,  $p = 0.049$ ) (Supplementary Tables S2 & S6). Interestingly, the ability to

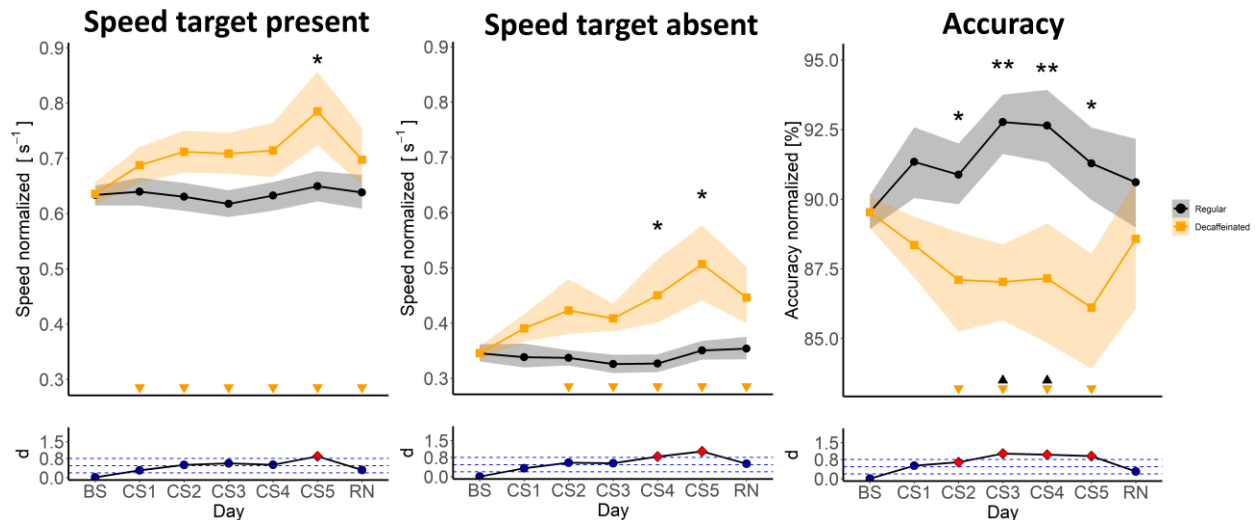


Figure 8: Evolution of orienting/selection across the experimental protocol. On each study day, performance on four visual search task (VST) sessions spread over the entire day were averaged. Lines and shaded areas represent means  $\pm$  95% confidence intervals in groups receiving either regular (black;  $n = 12$ ) or decaffeinated coffee (orange;  $n = 14$ ). Accuracy quantified the numbers of correct hits and correct rejections over total responses. Black (regular coffee group) and orange triangles (decaffeinated coffee group) above the x-axis represent Benjamini-Hochberg corrected differences ( $p_{corr} < 0.05$ ) from baseline (Supplementary Table S6). Stars indicate significant differences between the groups: \*\* $p < 0.005$ , \* $p < 0.05$ . The corresponding effect sizes (Cohen's  $d$ ) are represented underneath. Dashed blue lines separate regions of "small", "medium" or "large" effect size. Red dots indicate a significant group difference ( $p < 0.05$ ).

correctly recognize the target among the distractors was better than in baseline in the regular coffee group on restriction days 3 and 4 and impaired in the decaffeinated coffee group on restriction days 2 through 5. Thus, the regular coffee group performed more accurately throughout extended sleep restriction ('group':  $F_{1,26} = 8.11$ ,  $p = 0.008$ ; 'day' x 'group' interaction:  $F_{6,672} = 4.35$ ,  $p < 0.001$ ). All group differences were of medium to large effect size and no longer present after the recovery night.

### Visuo-spatial n-back task

Response speed and accuracy on the visuo-spatial n-back task depended on cognitive workload (speed:  $F_{2,2062} = 17.96$ ; accuracy:  $F_{2,2062} = 465.51$ ;  $p_{all} < 0.001$ ) and day of the protocol (speed:  $F_{6,2062} = 8.16$ ; accuracy:  $F_{6,2062} = 16.23$ ;  $p_{all} < 0.001$ ). Performance speed was faster than in baseline on many experimental days in the regular coffee group while it fell below the baseline level in the 1-back task on sleep restriction days 4 and 5 in the group receiving decaffeinated coffee (Fig. 9). Accuracy also deteriorated across sleep restriction on all cognitive workloads in the latter group whereas accuracy remained virtually unchanged in the former. Thus, the regular coffee group performed faster (except on the 3-back task) and/or more accurately than the decaffeinated coffee group on the majority of days during sleep restriction ('day' x 'group' interaction: speed:  $F_{6,2062} = 9.52$ ; accuracy:  $F_{6,2062} = 5.13$ ;  $p_{all} < 0.001$ ) (Supplementary Tables S3 & S7). The differences between the groups generally were of medium to large effect size and no longer present after the recovery night.

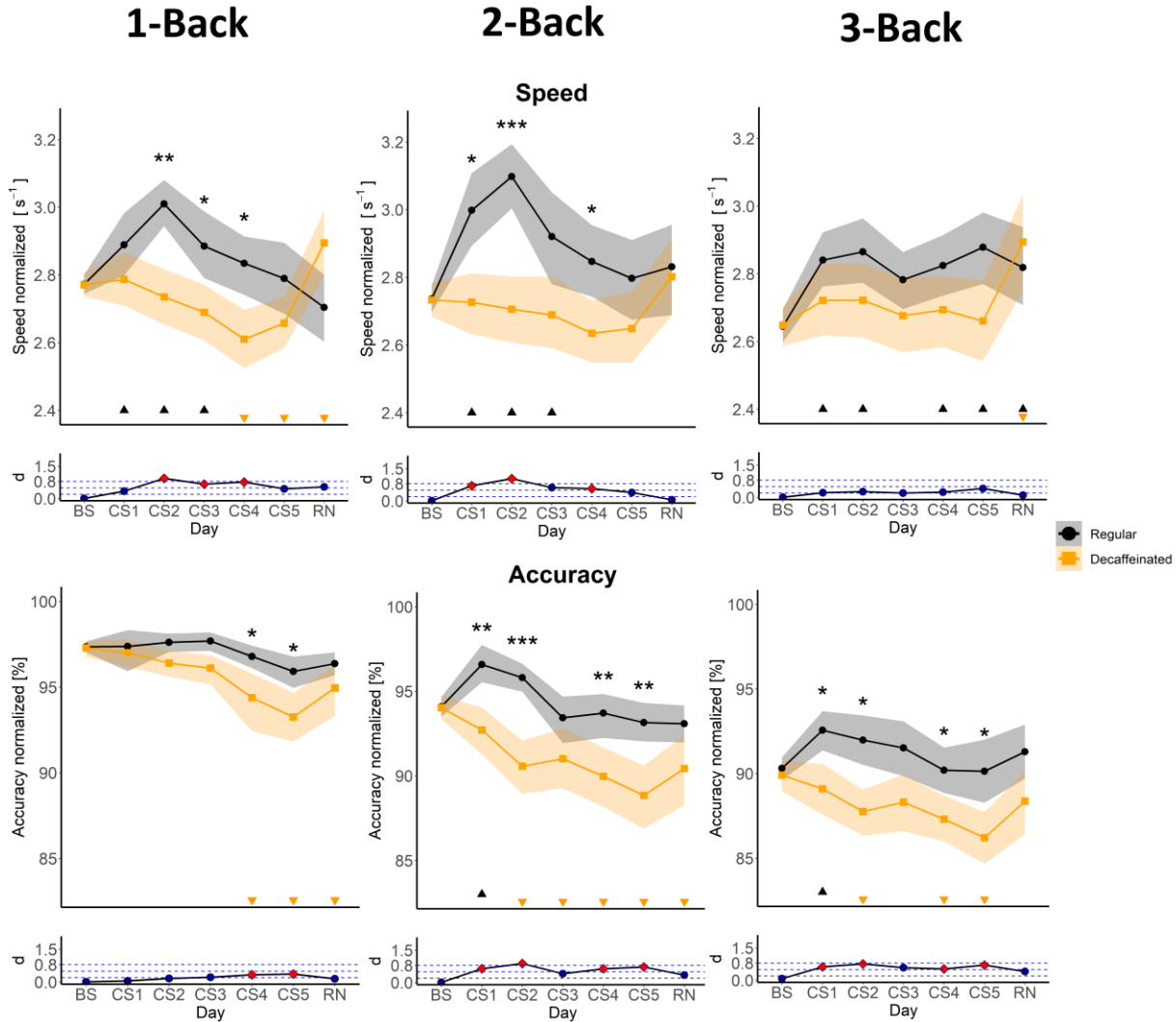


Figure 9: Evolution of orienting/selection across the experimental protocol. On each study day, performance on four visual search task (VST) sessions spread over the entire day were averaged. Lines and shaded areas represent means  $\pm$ 95% confidence intervals in groups receiving either regular (black;  $n = 12$ ) or decaffeinated coffee (orange;  $n = 14$ ). Accuracy quantified the numbers of correct hits and correct rejections over total responses. Black (regular coffee group) and orange triangles (decaffeinated coffee group) above the x-axis represent Benjamini-Hochberg corrected differences ( $p_{corr} < 0.05$ ) from baseline (Supplementary Table S6). Stars indicate significant differences between the groups:  $**p < 0.005$ ,  $*p < 0.05$ . The corresponding effect sizes (Cohen's  $d$ ) are represented underneath. Dashed blue lines separate regions of "small", "medium" or "large" effect size. Red dots indicate a significant group difference ( $p < 0.05$ ).

### Letter n-back task

The visuo-spatial n-back task alike, response speed and accuracy on the letter n-back task depended on cognitive workload (speed:  $F_{2,2065} = 191.32$ ; accuracy:  $F_{2,2065} = 285.36$ ;  $p_{all} < 0.001$ ) and day of the protocol (speed:  $F_{6,2065} = 6.43$ ; accuracy:  $F_{6,2065} = 9.82$ ;  $p_{all} < 0.001$ ). The effects of sleep restriction and coffee administration were similar to those on the visuo-spatial n-back task, except that speed also on the 3-back version of the task was enhanced on sleep restriction days 1 through 3 in the regular coffee group when compared to the decaffeinated coffee group (Fig. 10). The evolution of performance differed between the two groups ('day' x 'group')

interaction: speed:  $F_{6,2065} = 8.11$ ; accuracy:  $F_{6,2062} = 4.23$ ;  $p_{\text{all}} < 0.001$ ), such that the regular coffee group performed faster and/or more accurately than the decaffeinated coffee group on all 3 workload levels on many of sleep restriction days 1 through 4 (Supplementary Table S8). The differences between the groups generally were of large effect size, yet absent on sleep restriction day 5 and after the recovery night.

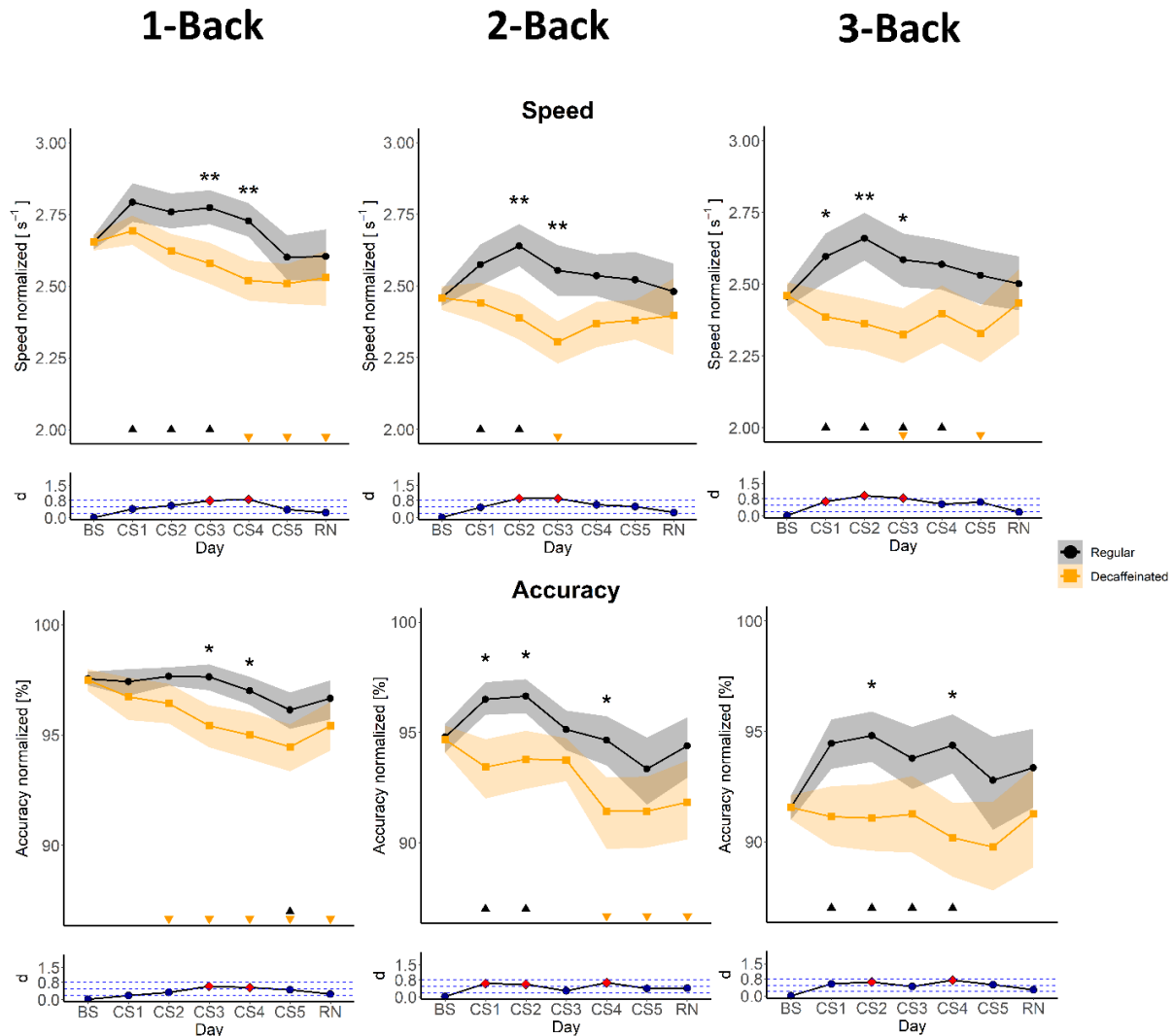


Figure 10: Evolution of visuo-spatial working memory and executive control across the experimental protocol. On each study day, performance on four *n*-back task sessions spread over the entire day were averaged. Lines and shaded areas represent means  $\pm 95\%$  confidence intervals in groups receiving either regular (black;  $n = 12$ ) or decaffeinated coffee (orange;  $n = 14$ ). Normalized mean speed and accuracy, calculated via sum of hits and correct rejections over total responses are shown for each step of cognitive load. Black (regular coffee group) and orange triangles (decaffeinated coffee group) above the x-axis represent Benjamini-Hochberg corrected differences ( $p_{\text{corr}} < 0.05$ ) from baseline (Supplementary Table S7). Stars indicate significant differences between the groups:  $***p < 0.005$ ,  $**p < 0.01$ ,  $*p < 0.05$ . The corresponding effect sizes (Cohen's *d*) are represented underneath. Dashed blue lines separate regions of "small", "medium" or "large" effect size. Red dots indicate a significant group difference ( $p < 0.05$ ).

## Caffeine and metabolites

Confirming that the study participants adhered to the instruction to abstain from all sources of caffeine prior to the experiment, caffeine and caffeine metabolites were undetectable at bedtime in both groups at baseline. Among the group receiving regular coffee, the mean caffeine levels increased until sleep restriction day 4, reaching a maximum of roughly 6  $\mu\text{mol/l}$  (Fig. 11). Afterwards, the concentration decreased because coffee was served only in the morning on sleep restriction day 5 and no coffee was administered after the recovery night. A similar time course, albeit less variable, was seen for the three primary caffeine metabolites, paraxanthine, theobromine, and theophylline. Very low concentrations of caffeine and metabolites were detected in saliva of the control group who received coffee '833', confirming that this batch contained only negligible amounts of caffeine ('day' x 'group' interaction:  $F_{6,112} \geq 10.01$ ,  $p_{\text{all}} < 0.001$ ; Supplementary Table S4).

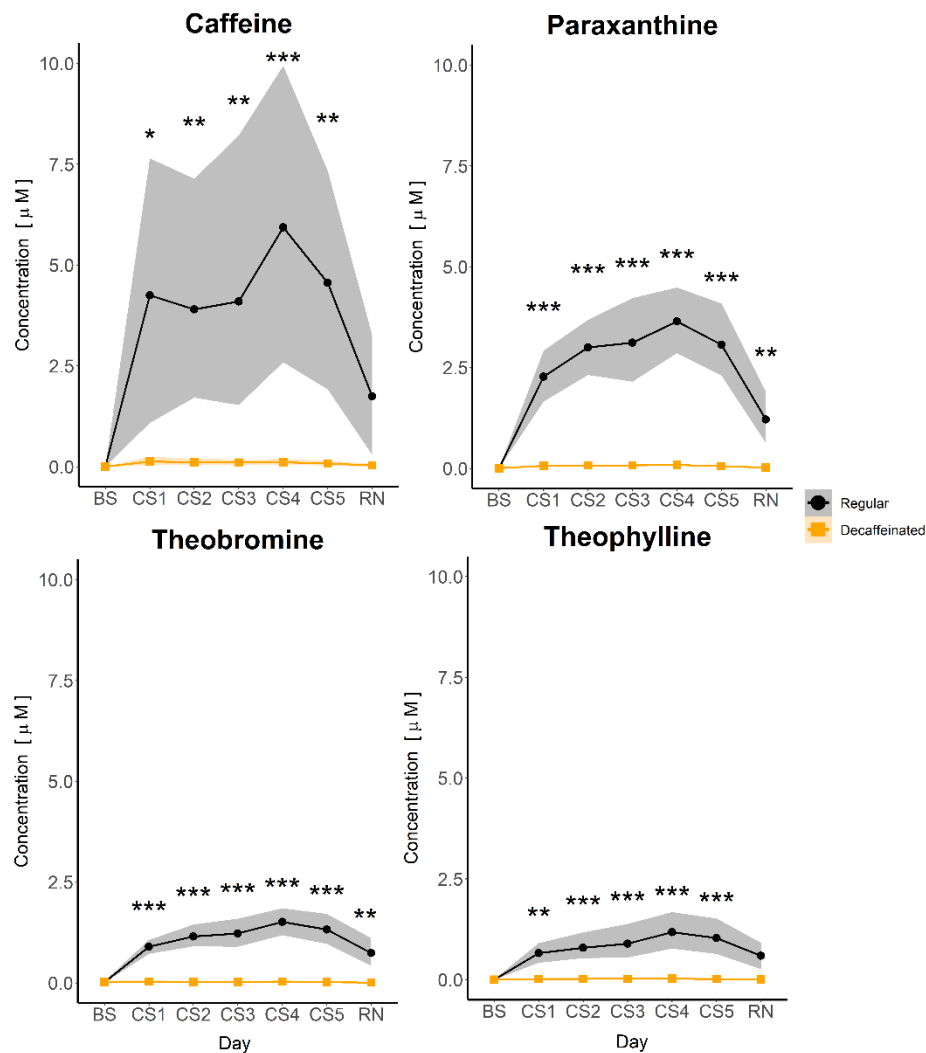


Figure 11: Evolution of caffeine and caffeine metabolite (paraxanthine, theobromine, and theophylline) concentrations in saliva at bedtime across the experimental protocol. The last portion of coffee was administered in the morning after CS5. The labels BS through RN refer to bedtimes of the respective sleep episodes, whereas in Figure 2, 3, 4 and 5 the labels refer to the wake periods after the respective sleep episodes. Lines and shaded areas represent means  $\pm$ 95% confidence intervals in groups receiving either regular (black;  $n = 10$ ) or decaffeinated coffee (orange;  $n = 11$ ). Stars indicate significant differences between the groups: \*\*\* $p < 0.005$ ; \*\* $p < 0.01$ , \* $p < 0.05$ .

## 2.5 Discussion

We addressed the question whether the prevalent habit of drinking morning and midday coffee and thus ingesting roughly 300 mg caffeine per day ensures optimal attention during chronic sleep restriction. Such a coffee intake pattern is common in Europe and South America. We found that regular coffee effectively attenuated the repercussions of five nights of time-in-bed restricted to 5 h on all three attentional domains (vigilance, orienting, executive control) when compared to decaffeinated coffee. Our data suggest that ‘real world’ coffee consumption is able to counteract – at least in part- attentional performance impairment in genetically caffeine sensitive adults due to repeated sleep restriction, which is common in many societies.

Our findings partly contrast with recent work suggesting that 2x200 mg caffeine per day loses efficacy to counteract the repercussions of sleep restriction on vigilance (Doty et al., 2017). In both studies, the evolution of subjective sleepiness, PVT speed, and PVT lapses across sleep restriction in the groups receiving placebo and decaffeinated coffee was highly similar. In addition, the slowing of mean PVT response speed in the active treatment groups was consistently attenuated in both experiments during the initial three restriction days. Afterwards, however, the findings diverged and in the prior report, tolerance to caffeine developed. In fact, the number of PVT lapses in the caffeine group was even higher than in the placebo group on sleep restriction days 4 and 5 (Doty et al., 2017). We observed no such tolerance and further performance impairment in the present experiment. By contrast, the number of lapses in the regular coffee group virtually remained close to the baseline level up until sleep restriction day 5 when a slight increase was observed. Together with more in-depth analyses of accuracy and cognitive information processing fidelity underlying PVT performance (Chavali et al., 2017), the data confirmed that regular coffee indeed provided a benefit over decaffeinated coffee and preserved task performance for 3-4 days of sleep restriction (Fig. 2). An important difference between the two studies is the prospective genotyping and selective enrollment of participants based on polymorphism rs5751875 of ADORA2A in the present experiment. The A2A receptor constitutes the primary target structure for caffeine effects on vigilance, attention, and sleep-wake regulation (Bodenmann et al., 2012; Huang et al., 2005; Renda et al., 2015; Rétey et al., 2007; Rupp et al., 2013). According to a new paradigm in the design of human studies to test the effects of caffeine and A2A receptor antagonists on sleep-wake processes and cognitive functions with decreased variability (Chen & Cunha, 2020; Holst et al., 2016; Satterfield et al., 2019), only homozygous C-allele carriers of ADORA2A here were examined. Supporting the notion that these individuals are sensitive to the effects of caffeine on vigilance and executive control in rested and sleep deprived state (Renda et al., 2015; Rétey et al., 2007), clear benefits of coffee on objective measures of attentional performance were found. By contrast, recent findings suggest that C-allele carriers of this polymorphism exhibit reduced interoceptive accuracy when compared to T-allele homozygotes (Geiger et al., 2016). It may be speculated that the reduced processing of interoceptive information in C-allele homozygotes underlies the lack of coffee effects on subjective sleepiness during repeated sleep restriction.

The orienting network may be relatively resilient against the detrimental effects of repeated sleep loss (Cunningham et al., 2018). Indeed, response speed on the visual search task was only moderately slowed in the decaffeinated coffee group and remained unaffected by sleep restriction in the regular coffee group. Furthermore, when performance accuracy was analyzed, we found a

consistent benefit of regular coffee over decaffeinated coffee (Fig. 3). This finding adds supporting evidence to the notion that caffeine can normalize attention when stressors dampen performance and that this benefit can persist for multiple days. In accordance with previous findings (Lo et al., 2012), both cognitive workload as well as sleep restriction reduced speed and accuracy on visuo-spatial and verbal n-back tasks. In general, regular coffee intake improved performance with medium to high effect size when compared to decaffeinated coffee. Similar to the effect on the alerting network, improved response speed on the visuo-spatial n-back task was restricted to the initial 3-4 sleep restriction days. Speed on the task with the highest cognitive workload (3-back) was the only metric probing executive control that was not improved by coffee. By contrast, task accuracy on all workload levels was boosted by coffee until the final day of sleep restriction (Fig. 4). The results on the letter n-back task basically corroborated these observations, yet the beneficial effects of coffee only lasted to the fourth sleep restriction day (Fig. 5). Together, the findings confirm the conclusions of previous work in rested individuals showing that caffeine increases executive control functions of the brain (Brunyé et al., 2010; Einöther & Giesbrecht, 2013). Functional imaging studies showed that caffeine up-regulates prefrontal brain areas in concert with anterior cingulate cortex that provide the executive control of visual attention (Koppelstaetter et al., 2008). Some evidence in mice suggests that caffeine-targeted A2A receptors control information flow in prefronto-cortical circuits by synergizing with dopamine D2 receptors (Real et al., 2018). Caffeine-induced increased dopaminergic neurotransmission in prefrontal cortex may thus support the executive functioning of the brain.

We intended to investigate the effects of habitual coffee intake which in dose and timing is similar to typical human behavior, as a countermeasure to impaired attention caused by prevailing sleep restriction. To standardize the experimental conditions and to avoid withdrawal reactions in the decaffeinated group, all study participants abstained from caffeine intake for at least 10 days prior to the first sleep restriction day. However, in the “real world”, most people consume caffeinated beverages every day, regardless of prior partial sleep loss. Although still a matter of discussion, findings in rats and mice indicate that chronic caffeine intake increases the number of adenosine receptors and their sensitivity both *in vitro* as well as *in vivo*, and caffeine withdrawal decreases locomotor activity in rats for several days (reviewed by Nehlig et al., 1992). In humans, the regulation of cerebral adenosine receptors by chronic caffeine and changes in attentional performance over multiple days after caffeine abstinence in habitual moderate consumers have not been explored. It is intriguing to note that in the present study, regular coffee improved several attentional measures, particularly speed on the PVT and the n-back tasks, during the first two to three sleep restriction days when compared to baseline. This observation could indicate that due to a highly sensitive adenosine system, the re-introduction of caffeine not only attenuated the impairment by sleep restriction, but initially improved baseline performance despite increasing sleep debt. Future research is warranted to study the possible underpinnings of this unexpected observation.

The caffeine and metabolite levels in saliva confirmed that the decaffeinated coffee contained negligible amounts of caffeine. The concentrations of the caffeine metabolites in the regular coffee group reflect the different ratios at which they are formed in the liver (Camandola et al., 2019). Among many other phytochemicals, coffee contains theobromine and theophylline, whereas paraxanthine is not present in plant extracts (Camandola et al., 2019). The psychostimulant effects of coffee are commonly attributed to caffeine, which acutely affects neuronal network

activity and promotes alertness and attention over a wide dose range (McLellan et al., 2016). In humans, caffeine is metabolized in the liver through the cytochrome P450 isoenzyme, CYP1A2, which accounts for almost all primary metabolism of caffeine.

The CYP1A2 enzymatic activity exhibits pronounced inter-individual variation, likely underlying the large variation in individual saliva caffeine concentrations (Fig. 6). Paraxanthine is formed by demethylation of caffeine. Roughly 10 h after coffee intake, the paraxanthine concentration reaches levels comparable to or even higher than those of caffeine (Camandola et al., 2019; Urry et al., 2016). The circulating concentrations of both these methylxanthines were sufficient to block adenosine receptors (Müller & Jacobson, 2011). Both chemicals should be considered when interpreting the beneficial effects of coffee consumption against the detrimental consequences of repeated sleep restriction on vigilance and attention. On the other hand, caffeine easily crosses the blood-brain barrier, whereas much less paraxanthine enters the brain (Camandola et al., 2019). Because saliva was only sampled at bedtime, whereas sleepiness and cognitive performance were tested throughout the day, it is not possible to reliably estimate the respective contributions of sleep restriction, caffeine, and caffeine metabolites on the observed time courses of individual behavioral changes across the

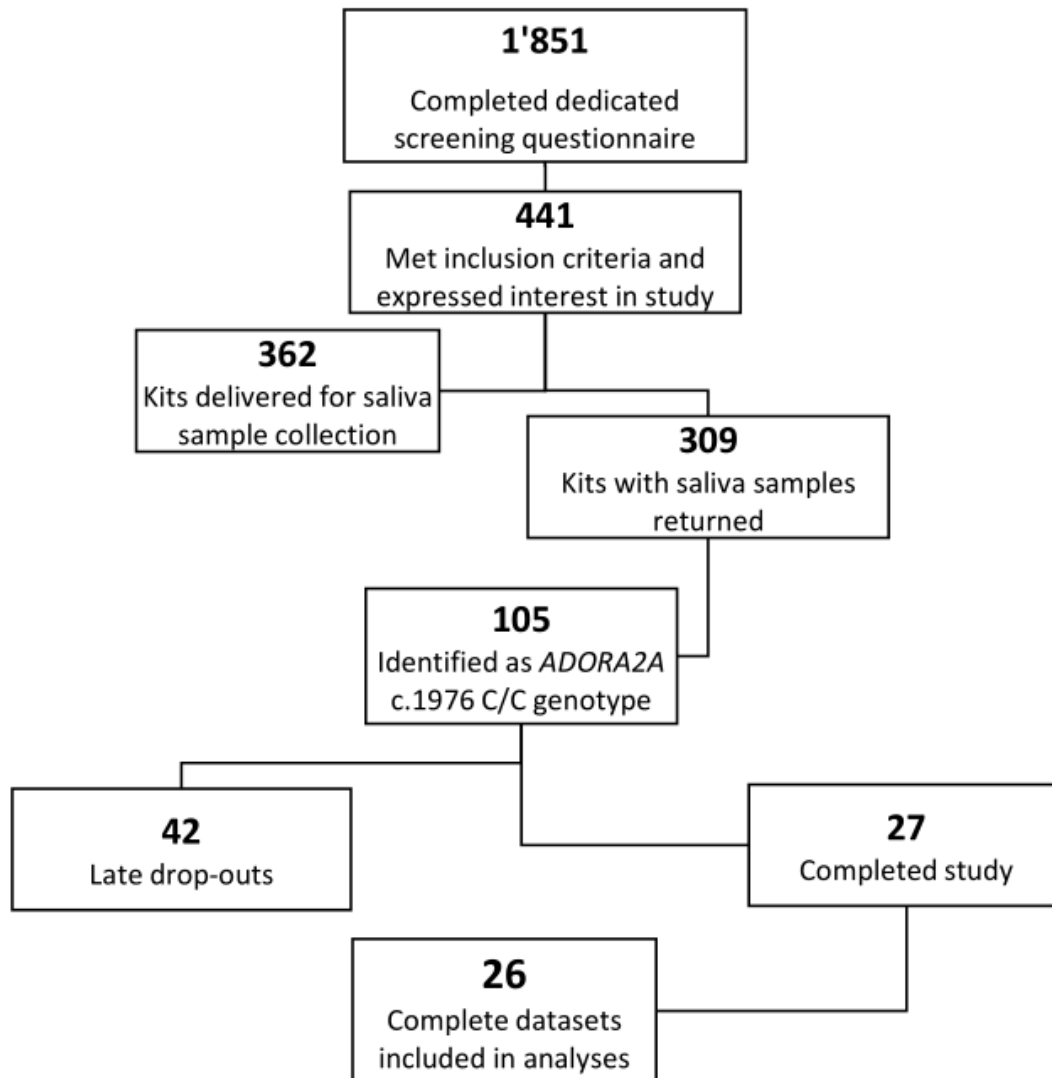
sleep restriction. Future studies for that purpose are warranted.

In conclusion, we found that daily 300 mg caffeine intake in coffee effectively reduced impairments of vigilance and attention across five days/nights of sleep restriction in genetically caffeine sensitive individuals. The C/C genotype of ADORA2A is present in roughly 35% of individuals in European populations. The selective enrollment of this genotype thus limits the generalizability of the present results. Nevertheless, the findings support the conclusion that dietary inhibition of adenosine A2A receptors can persistently benefit vigilance and attention during repeated sleep restriction. The coffee-induced blockade of these receptors may potentiate cholinergic and monoaminergic neurotransmission in thalamus, anterior cingulate and other cortical regions that regulate vigilance, alerting and executive control (Fan et al., 2005). In ongoing analyses of our data that will be published elsewhere, we examine whether the repeated coffee consumption also affects the consequences of sleep restriction on reversal learning decision making (Whitney et al., 2015) and risk taking (Maric et al., 2017). In addition, we investigate whether this highly prevalent behavior attenuates or accelerates the evolution of waking and sleep electroencephalographic markers of sleep need across sleep curtailment and recovery sleep (Landolt et al., 2004), as well as possible changes in the availability of cerebral adenosine A1 receptors (Elmenhorst et al., 2018).

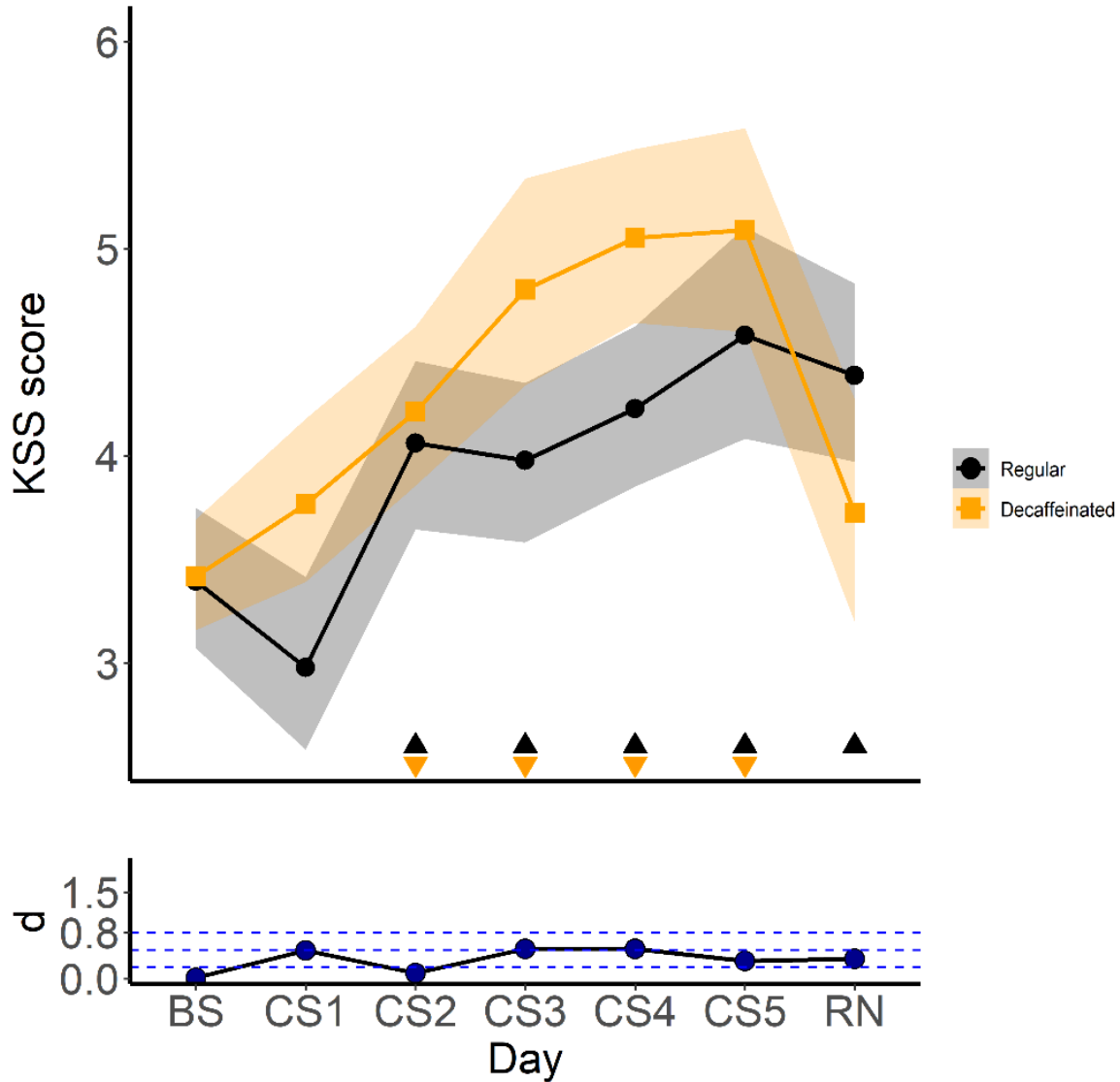
## 2.6 Supplementary material

### Supplementary Figure 1

Flow chart of volunteer recruitment



# Karolinska Sleepiness Scale (KSS)



Supplementary Figure S2: Evolution of subjective sleepiness across the experimental protocol. On each study day, 4 assessments of the Karolinska Sleepiness Scale (KSS) spread over the entire day were averaged. Lines and shaded areas represent means  $\pm$  95% confidence intervals in the groups receiving either regular (black;  $n = 12$ ) or decaffeinated coffee (orange;  $n = 14$ ). The bottom panel represents the effect size (Cohen's  $d$ ) of the group difference. Dashed blue lines, separate regions of "small", "medium" or "large" effect size. Blue dots indicate a non-significant group difference ( $p_{all} > 0.05$ ). Black (regular coffee group) and orange triangles (decaffeinated coffee group) above the x-axis represent Benjamini-Hochberg corrected differences ( $p_{corr} < 0.05$ ) from baseline (Table S1).

Supplementary Table 1: Statistical analysis of subjective sleepiness and psychomotor vigilance test.

Linear mixed effects model analysis of variance of data derived from Karolinska Sleepiness Scale (KSS) and psychomotor vigilance test (PVT) with 'day' (BL, CS1, CS2, CS3, CS4, CS5, RN) and 'group' (regular vs. decaffeinated) as fixed effects, and 'subject' as random effect. Significant p-values are highlighted in bold.

Variable		Day	Group	Day x Group
<b>KSS</b>	Sum of squares	197.91	2.47	41.13
	Mean square	32.98	2.47	6.85
	Degrees of freedom	6, 672	1, 26	6, 672
	F value	17.50	1.31	3.64
	p value	<b>&lt;0.001</b>	0.263	<b>0.001</b>
<b>PVT: Speed</b>	Sum of squares	7.04	0.07	1.27
	Mean square	1.17	0.07	0.21
	Degrees of freedom	6, 672	1, 26	6, 672
	F value	42.90	2.41	7.72
	p value	<b>&lt;0.001</b>	0.133	<b>&lt;0.001</b>
<b>PVT: Lapses</b>	Sum of squares	812.53	25.26	157.88
	Mean square	135.42	25.26	26.31
	Degrees of freedom	6, 672	1, 26	6, 672
	F value	19.00	3.54	3.69
	p value	<b>&lt;0.001</b>	0.071	<b>0.001</b>
<b>PVT: Accuracy</b>	Sum of squares	826.61	36.39	269.97
	Mean square	137.77	36.39	45
	Degrees of freedom	6, 672	1, 26	6, 672
	F value	13.84	3.66	4.52
	p value	<b>&lt;0.001</b>	0.067	<b>&lt;0.001</b>
<b>PVT: signal-to-noise ratio</b>	Sum of squares	41.47	4.15	49.51
	Mean square	6.91	4.15	8.25
	Degrees of freedom	6, 672	1, 26	6, 672
	F value	7.99	4.80	9.54
	p value	<b>&lt;0.001</b>	<b>0.038</b>	<b>&lt;0.001</b>

Supplementary Table 2: Statistical analysis of the visual search task. Linear mixed effects model analysis for visual search task performance, with 'day' (BL, CS1, CS2, CS3, CS4, CS5, RN) and 'group' (regular vs. decaffeinated) as fixed effects; 'subject' and 'set size of distractors' as random effect. Significant p-values are highlighted in bold.

Variable		Day	Group	Day x Group
<b>Speed target present</b>	Sum of squares	1.47	0.19	1.06
	Mean square	0.24	0.19	0.18
	Degrees of freedom	6, 2759	1, 26	6, 2759
	F value	4.26	3.28	3.08
	p value	<b>&lt;0.001</b>	0.082	<b>0.005</b>
<b>Speed target absent</b>	Sum of squares	1.76	0.23	1.53
	Mean square	0.29	0.23	0.26
	Degrees of freedom	6, 2759	1, 26	6, 2759
	F value	5.56	4.29	4.83
	p value	<b>&lt;0.001</b>	<b>0.049</b>	<b>&lt;0.001</b>
<b>Accuracy</b>	Sum of squares	77	135.5	436.55
	Mean square	12.83	135.5	72.76
	Degrees of freedom	6, 672	1, 26	6, 672
	F value	0.77	8.11	4.35
	p value	0.595	<b>0.008</b>	<b>&lt;0.001</b>

Supplementary Table 3: Statistical analysis of the visuo-spatial and letter n-back tasks. Linear mixed effects model analysis of visuo-spatial and letter N-back task performance, with 'day' (BL, CS1, CS2, CS3, CS4, CS5, RN) and 'group' (regular vs. decaffeinated) as fixed effects, and 'subject' as random effect. Significant p-values are highlighted in bold.

Variable		Day	Group	Workload (n)	Day x Group	N x Group	N x Group x Day
<b>Spatial n-back task</b>							
Speed	Sum of squares	5.10	0.79	3.75	5.96	0.85	1.33
	Mean square	0.85	0.79	1.87	0.99	0.43	0.11
	Degrees of freedom	6, 2062	1, 26	2, 2062	6, 2062	2, 2062	12, 2062
	F value	8.16	7.59	17.96	9.52	4.09	1.06
	p value	<b>&lt;0.001</b>	<b>0.011</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.017</b>	0.386
Accuracy	Sum of squares	1970.70	318.20	18846.90	622.90	333.90	190.70
	Mean square	328.40	318.20	9423.4	103.8	167	15.9
	Degrees of freedom	6, 2062	1, 26	2, 2062	6, 2062	2, 2062	12, 2062
	F value	16.23	15.72	465.51	5.13	8.25	0.79
	p value	<b>&lt;0.001</b>	<b>0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.667
<b>Letter n-back task</b>							
Speed	Sum of squares	2.55	0.73	25.31	3.22	0.28	0.45
	Mean square	0.43	0.73	12.66	0.54	0.14	0.04
	Degrees of freedom	6, 2065	1, 26	2, 2065	6, 2065	2, 2065	12, 2065
	F value	6.43	11.06	191.32	8.11	2.14	0.57
	p value	<b>&lt;0.001</b>	<b>0.003</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	0.118	0.869
Accuracy	Sum of squares	1004.20	180.60	9725.50	432.40	170.20	138.40
	Mean square	167.40	180.60	4862.8	72.1	85.1	11.5
	Degrees of freedom	6, 2065	1, 26	2, 2065	6, 2065	2, 2065	12, 2065
	F value	9.82	10.60	285.36	4.23	4.99	0.68
	p value	<b>&lt;0.001</b>	<b>0.003</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.007</b>	0.775

Supplementary Table 4: Statistical analysis of caffeine and the caffeine metabolites, paraxanthine, theobromine, and theophylline in saliva. Linear mixed effects model analysis for caffeine and metabolite quantification with 'day' (BL, CS1, CS2, CS3, CS4, CS5, RN) and 'group' (regular vs. decaffeinated) as fixed effects, and 'subject' as random effect. Significant p-values are highlighted in bold.

<b>Molecule</b>		<b>Day</b>	<b>Group</b>	<b>Day x Group</b>
<b>Caffeine</b>	Sum of squares	136.16	23.10	126.00
	Mean square	22.69	23.10	21
	Degrees of freedom	6, 112	1, 21	6, 112
	F value	10.81	11.01	10.01
	p value	<b>&lt;0.001</b>	<b>0.003</b>	<b>&lt;0.001</b>
<b>Paraxanthine</b>	Sum of squares	56.55	14.47	51.81
	Mean square	9.42	14.47	8.64
	Degrees of freedom	6, 112	1, 21	6, 112
	F value	45.61	70.01	41.79
	p value	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<b>Theobromine</b>	Sum of squares	8.17	2.58	8.12
	Mean square	1.36	2.58	1.35
	Degrees of freedom	6, 112	1, 21	6, 112
	F value	37.26	70.44	37.01
	p value	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
<b>Theophylline</b>	Sum of squares	5.09	1.10	4.70
	Mean square	0.85	1.10	0.78
	Degrees of freedom	6, 112	1, 21	6, 112
	F value	20.81	26.89	19.25
	p value	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>

Supplementary Table 5: Group comparisons, Karolinska Sleepiness Scale and psychomotor vigilance test.

Variable	Day	Estimate	SE	t ratio	p-value corr
KSS	BS	-0.11	0.36	-0.31	0.084
	CS1	-0.79	0.4	-1.98	0.432
	CS2	-0.15	0.4	-0.38	0.083
	CS3	-0.82	0.4	-2.07	0.452
	CS4	-0.82	0.4	-2.07	0.452
	CS5	-0.49	0.4	-1.24	0.269
	RN	0.69	0.43	1.59	0.298
PVT: Speed	BS	0	0.06	0.01	0.996
	CS1	0.15	0.06	2.58	<b>0.029</b>
	CS2	0.18	0.06	2.97	<b>0.029</b>
	CS3	0.16	0.06	2.63	<b>0.029</b>
	CS4	0.1	0.06	1.64	0.188
	CS5	0.03	0.06	0.48	0.742
	RN	-0.08	0.06	-1.35	0.255
PVT: Lapses	BS	-0.01	0.86	-0.01	0.995
	CS1	-0.28	0.86	-0.33	0.865
	CS2	-0.53	0.86	-0.62	0.751
	CS3	-2.42	0.86	-2.83	<b>0.022</b>
	CS4	-2.47	0.86	-2.88	<b>0.022</b>
	CS5	-1.76	0.86	-2.06	0.101
	RN	-1.43	0.91	-1.56	0.213
PVT: Accuracy	BS	0.01	1	0.01	0.992
	CS1	0.51	1	0.51	0.852
	CS2	0.23	1	0.23	0.956
	CS3	3.02	1	3.02	<b>0.012</b>
	CS4	3.27	1	3.26	<b>0.013</b>
	CS5	2.02	1	2.02	0.111
	RN	1.45	1.07	1.36	0.31
PVT: LSNR	BS	0.66	0.54	1.23	0.263
	CS1	1.31	0.54	2.43	<b>0.047</b>
	CS2	1.73	0.54	3.23	<b>0.011</b>
	CS3	1.7	0.54	3.16	<b>0.011</b>
	CS4	1.18	0.54	2.2	0.06
	CS5	0.94	0.54	1.55	0.125
	RN	0	0.55	-0.01	0.994

Supplementary Table 6: Group comparisons, visual search task.

Variable	Day	Estimate	SE	t ratio	p-value corr
Speed target present	BS	0	0.05	-0.04	0.971
	CS1	-0.05	0.05	-1	0.374
	CS2	-0.08	0.05	-1.76	0.148
	CS3	-0.09	0.05	-1.97	0.148
	CS4	-0.08	0.05	-1.77	0.148
	CS5	-0.14	0.05	-2.94	<b>0.034</b>
	RN	-0.06	0.05	-1.16	0.348
Speed target absent	BS	0	0.05	0	0.997
	CS1	-0.05	0.05	-1.09	0.327
	CS2	-0.09	0.05	-1.82	0.122
	CS3	-0.08	0.05	-1.74	0.122
	CS4	-0.12	0.05	-2.62	<b>0.041</b>
	CS5	-0.16	0.05	-3.3	<b>0.013</b>
	RN	-0.09	0.05	-1.84	0.122
Accuracy	BS	0	1.27	0	0.997
	CS1	2.47	1.28	1.94	0.079
	CS2	3.21	1.27	2.52	<b>0.024</b>
	CS3	4.55	1.27	3.59	<b>0.002</b>
	CS4	4.52	1.27	3.56	<b>0.002</b>
	CS5	3.72	1.27	2.92	<b>0.011</b>
	RN	1.17	1.36	0.86	0.457

Supplementary Table 7: Group comparisons, visuo-spatial n-back task.

Variable	Day	Estimate	SE	t ratio	p-value corr
<b>Speed 1-back</b>	<b>BS</b>	0	0.08	0.03	0.98
	<b>CS1</b>	0.1	0.08	1.24	0.258
	<b>CS2</b>	0.27	0.08	3.36	<b>0.009</b>
	<b>CS3</b>	0.2	0.08	2.39	<b>0.046</b>
	<b>CS4</b>	0.22	0.08	2.74	<b>0.028</b>
	<b>CS5</b>	0.13	0.08	1.64	0.155
	<b>RN</b>	-0.19	0.09	-1.92	<b>0.091</b>
<b>Speed 2-back</b>	<b>BS</b>	0	0.09	0.04	0.972
	<b>CS1</b>	0.27	0.09	2.82	<b>0.021</b>
	<b>CS2</b>	0.39	0.09	4.13	<b>0.001</b>
	<b>CS3</b>	0.21	0.09	2.24	0.079
	<b>CS4</b>	0.23	0.09	2.46	<b>0.048</b>
	<b>CS5</b>	0.15	0.09	1.55	0.175
	<b>RN</b>	0.02	0.1	0.23	0.958
<b>Speed 3-back</b>	<b>BS</b>	0	0.08	-0.04	0.968
	<b>CS1</b>	0.12	0.08	1.48	0.243
	<b>CS2</b>	0.14	0.08	1.79	0.238
	<b>CS3</b>	0.11	0.08	1.34	0.255
	<b>CS4</b>	0.13	0.08	1.64	0.238
	<b>CS5</b>	0.22	0.08	1.74	0.165
	<b>RN</b>	-0.08	0.09	-0.85	0.461
<b>Accuracy 1-back</b>	<b>BS</b>	0.07	0.72	0.09	0.928
	<b>CS1</b>	0.37	0.73	0.5	0.717
	<b>CS2</b>	1.21	0.73	1.66	0.136
	<b>CS3</b>	1.58	0.72	2.18	0.069
	<b>CS4</b>	2.2	0.72	2.62	<b>0.034</b>
	<b>CS5</b>	2.17	0.73	2.46	<b>0.02</b>
	<b>RN</b>	1.42	0.84	1.7	0.136
<b>Accuracy 2-back</b>	<b>BS</b>	0.05	1.28	0.04	0.966
	<b>CS1</b>	3.84	1.29	2.98	<b>0.008</b>
	<b>CS2</b>	5.22	1.29	4.05	<b>0.001</b>
	<b>CS3</b>	2.42	1.28	1.88	0.074
	<b>CS4</b>	3.74	1.28	2.91	<b>0.008</b>
	<b>CS5</b>	4.29	1.29	3.33	<b>0.004</b>
	<b>RN</b>	2.63	1.4	1.87	0.074
<b>Accuracy 3-back</b>	<b>BS</b>	0.4	1.35	0.3	0.768
	<b>CS1</b>	3.49	1.35	2.58	<b>0.027</b>
	<b>CS2</b>	4.21	1.35	3.12	<b>0.017</b>
	<b>CS3</b>	2.23	1.35	2.09	0.083
	<b>CS4</b>	3.21	1.35	2.39	<b>0.047</b>
	<b>CS5</b>	3.91	1.35	2.9	<b>0.017</b>
	<b>RN</b>	2.84	1.46	1.95	0.063

Supplementary Table 8: Group comparisons, letter n-back task.

Variable	Day	Estimate	SE	t ratio	p-value corr
<b>Speed 1-back</b>	<b>BS</b>	0	0.06	0	0.998
	<b>CS1</b>	0.1	0.06	1.64	0.18
	<b>CS2</b>	0.14	0.06	2.27	0.061
	<b>CS3</b>	0.19	0.06	3.18	<b>0.007</b>
	<b>CS4</b>	0.21	0.06	3.4	<b>0.007</b>
	<b>CS5</b>	0.09	0.06	1.54	0.18
	<b>RN</b>	0.07	0.07	1.08	0.33
<b>Speed 2-back</b>	<b>BS</b>	0	0.07	0.01	0.989
	<b>CS1</b>	0.13	0.07	1.83	0.099
	<b>CS2</b>	0.25	0.07	3.46	<b>0.004</b>
	<b>CS3</b>	0.25	0.07	3.41	<b>0.004</b>
	<b>CS4</b>	0.17	0.07	2.28	0.059
	<b>CS5</b>	0.14	0.07	1.97	0.093
	<b>RN</b>	0.08	0.08	1.04	0.351
<b>Speed 3-back</b>	<b>BS</b>	0	0.09	-0.04	0.968
	<b>CS1</b>	0.21	0.09	2.45	<b>0.039</b>
	<b>CS2</b>	0.3	0.09	3.44	<b>0.007</b>
	<b>CS3</b>	0.26	0.09	3.03	<b>0.012</b>
	<b>CS4</b>	0.17	0.09	1.99	0.072
	<b>CS5</b>	0.2	0.09	1.34	0.099
	<b>RN</b>	0.07	0.09	0.73	0.543
<b>Accuracy 1-back</b>	<b>BS</b>	0.06	0.71	0.08	0.933
	<b>CS1</b>	0.69	0.72	0.97	0.39
	<b>CS2</b>	1.21	0.71	1.69	0.162
	<b>CS3</b>	2.22	0.71	3.12	<b>0.016</b>
	<b>CS4</b>	2.01	0.71	2.82	<b>0.019</b>
	<b>CS5</b>	1.65	0.71	2.11	0.073
	<b>RN</b>	1.21	0.78	1.55	0.174
<b>Accuracy 2-back</b>	<b>BS</b>	0.1	1.09	0.09	0.927
	<b>CS1</b>	3.08	1.1	2.8	<b>0.022</b>
	<b>CS2</b>	2.87	1.1	2.62	<b>0.024</b>
	<b>CS3</b>	1.38	1.09	1.26	0.246
	<b>CS4</b>	3.21	1.09	2.94	<b>0.022</b>
	<b>CS5</b>	1.92	1.1	1.75	0.116
	<b>RN</b>	2.52	1.19	1.89	0.093
<b>Accuracy 3-back</b>	<b>BS</b>	0.01	1.4	0.01	0.992
	<b>CS1</b>	3.29	1.41	2.34	0.052
	<b>CS2</b>	3.74	1.4	2.66	<b>0.032</b>
	<b>CS3</b>	2.53	1.4	1.81	0.104
	<b>CS4</b>	4.19	1.4	3	<b>0.025</b>
	<b>CS5</b>	3.02	1.4	1.86	0.099
	<b>RN</b>	2.06	1.51	1.36	0.205

# 3 Concentration-effect relationship of plasma caffeine on EEG delta power and cardiac autonomic activity during human NREM sleep

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### 3.1 Abstract

Acute caffeine intake affects brain and cardiovascular physiology, yet the concentration-effect relationships on the electroencephalogram (EEG) and cardiac autonomic activity during sleep are poorly understood. To tackle this question, we simultaneously quantified the plasma caffeine concentration with ultra-high-performance liquid chromatography, as well as the EEG, heart rate and high-frequency (0.15-0.4 Hz) spectral power in heart rate variability (HR-HRV), representing parasympathetic activity, with standard polysomnography during undisturbed human sleep. Twenty-one healthy young men ingested in randomized, double-blind, cross-over fashion, 160 mg caffeine or placebo in a delayed, pulsatile-release caffeine formula at their habitual bedtime, and initiated a four-hour sleep opportunity 4.5 hours later. The mean caffeine levels during sleep exhibited high individual variability between 0.2 and 18.4  $\mu\text{mol/l}$ . Across the first two NREM-REM sleep cycles, EEG delta (0.75-2.5 Hz) activity and heart rate were reliably modulated by waking and sleep states. Caffeine dose-dependently reduced delta activity and heart rate, and increased HR-HRV in NREM sleep when compared to placebo. The average reduction in heart rate equaled  $3.24 \pm 0.77$  beats per minute. Non-linear statistical models suggest that caffeine levels above  $\sim 7.4 \mu\text{mol/l}$  decreased EEG delta activity, whereas concentrations above  $\sim 4.3 \mu\text{mol/l}$  and  $\sim 4.9 \mu\text{mol/l}$ , respectively, reduced heart rate and increased HR-HRV. The findings provide quantitative concentration-effect relationships of caffeine, EEG delta power and cardiac autonomic activity and suggest increased parasympathetic activity during sleep after intake of caffeine.

### 3.2 Introduction

An oral dose of caffeine is rapidly absorbed, easily crosses the blood brain barrier, and affects sleep when taken shortly before bedtime (Bonati et al., 1982; Gardiner et al., 2023). It is widely accepted that the effects of moderate caffeine intake on sleep are mediated by competitive antagonism of adenosine A1 and A2A receptors in the central nervous system (Lazarus, Oishi, et al., 2019; Reichert et al., 2022b). Apart from the typical changes in sleep architecture (Clark and Landolt 2017), acute caffeine consistently attenuates EEG activity in the delta range ( $< 4.5$  Hz) in non-rapid-eye-movement (NREM) sleep (Carrier et al., 2009; Drake et al., 2013; Landolt, Dijk, et al., 1995; Van Dongen et al., 2001). Interestingly, several studies demonstrated that caffeine concentrations deemed too low to exert an acute pharmacological response are followed by reduced low-frequency activity ( $< 2$  Hz) during subsequent sleep (Landolt et al., 2004; Landolt, Werth, et al., 1995; Rétey et al., 2007). Thus, it is currently unknown whether caffeine concentrations below the limit of detection, an attenuated build-up of sleep pressure by blocking adenosine receptors during wakefulness, or active metabolites such as paraxanthine underpin caffeine's effect on sleep intensity (Reichert et al., 2022b).

Apart from regulating the adaptive homeostatic response to the prior duration of sleep and wakefulness on the sleep EEG, adenosine also contributes to the regulation of other major physiological processes. For example, by acting on all known subtypes of adenosine receptors, adenosine elicits a complex hemodynamic response that reflects separate effects on the

myocardium, the vascular tone, and the sympathetic nervous system (for review see Riksen et al., 2011). Exogenous adenosine administration to conscious humans elicits a consistent increase in heart rate and systolic blood pressure, and a drop in diastolic blood pressure (Riksen et al., 2011). On the other hand, the effects of caffeine on heart rate and blood pressure are typically small and differ widely among studies (Turnbull et al., 2017). Acute exposure to caffeine doses contained in 2-3 cups of coffee (> 200-300 mg) typically stimulates a transient increase in systolic and diastolic blood pressure, whereas both bradycardia and tachycardia were reported (Crooks et al., 2019; Riksen et al., 2011; Temple et al., 2017; Turnbull et al., 2017). Methylxanthine intoxication induces hypotension and tachyarrhythmias (Whitsett et al., 1984). The divergent findings are likely related to the different doses administered, but also reflect genetic predisposition, caffeine intake habits and health status of study participants, as well as other possible influences (Crooks et al., 2019; Green et al., 1996; Koenig et al., 2013). They may also suggest that the blockade of adenosine receptors only plays a minor role in mediating the actions of caffeine on the cardiovascular system (Fredholm et al., 2017). At least at higher doses, increased intracellular calcium, release of norepinephrine, and dopamine receptor sensitization constitute other likely mechanisms of action of caffeine on cardiovascular functions (Temple et al., 2017).

We recently reported the development of a pulsatile-release caffeine formulation to attenuate symptoms of sleep inertia after sleep restriction (Dornbierer et al., 2021). Following low-dose (160 mg) caffeine intake in an engineered capsule at habitual bedtime, we observed a mean caffeine plasma concentration of ~ 5 µmol/l at the beginning of a 4-hour sleep opportunity. We used this unique data set, to simultaneously quantify the evolution of caffeine levels, sleep architecture and the sleep EEG, as well as heart rate and the high-frequency component of the heart-rate variability (HF-HRV) spectrum as a marker of parasympathetic activity during sleep. Using generalized additive models, we aimed at establishing the concentration-effect relationships between caffeine in blood plasma, EEG low-frequency activity and cardiac autonomic activity in NREM sleep.

### 3.3 Methods

#### **Permission**

The study was approved by the Cantonal Ethics Committee of the Canton of Zurich (BASEC: 2018-00533) and registered on ClinicalTrials.gov (Identifier: NCT04975360). All participants provided written informed consent according to the declaration of Helsinki.

#### **Participants**

Twenty-two healthy young men (mean age: 23.8 ± 3.0 years; range: 19–30 years) with a body mass index between 19.4 and 25.3 kg m<sup>-2</sup> (mean: 22.6 ± 1.6 kg m<sup>-2</sup>) completed the study. They met the following inclusion criteria: habitual caffeine consumption of less than four regular units per day (coffee, tea, chocolate, cola, energy drinks); male sex to avoid the impact of menstrual cycle on physiology during sleep; age between 18 and 34 years; body mass index between 20 and 25 kg m<sup>-2</sup>; Epworth Sleepiness Score below 10; habitual sleep-onset latency below 20 min (self-rated); regular sleep–wake rhythm with bedtime between 22:00 hours and 01:00 hours; absence of any somatic or psychiatric disorders; no acute or chronic medication intake; non-

smoker; and no history of drug abuse (lifetime use < 5, with exception of occasional cannabis use). The participants were instructed to abstain from illicit drugs and caffeine during the entire study, starting 2 weeks prior to the first experimental night until the end of the study (the day after the second experimental night). No alcohol was allowed 24 hr before the experimental nights. The minimal washout period of caffeine between experimental nights was 7 days. Approximately 2 weeks ( $15 \pm 3$  days) prior to the first experimental night and lasting until the end of the study, participants were instructed to keep a regular sleep–wake rhythm, consistent with the volunteers' habitual bedtime. To verify adherence to the regular sleep and wake times, participants wore a rest–activity monitor on the non-dominant arm and kept a sleep–wake diary. All included participants showed roughly a 22:00 hours–06:00 hours ( $n = 2$ ), 23:00 hours–07:00 hours ( $n = 17$ ) or 00:00 hours–08:00 hours ( $n = 2$ ) rest period. During the pre-study period, participants on average went to bed at 23:10 hours  $\pm$  35 min, rose at 07:11 hours  $\pm$  30 min, and rested 8.0 hr  $\pm$  19 min per night. To facilitate the readability of the manuscript, clock times in the text and figures refer to the majority of participants who adhered to the 23:00 hours–07:00 hours sleep schedule.

### **Study design**

All participants spent two nights in the sleep laboratory, separated by at least one week. Upon arrival between 20:00-21:00, participants were informed in detail about the study protocol. Afterwards, the electrodes for polysomnographic recordings were carefully placed according to standard criteria (Berry et al., 2017) and the venous catheter for continuous blood collection was applied. To ensure sleep restriction and prevent unintended sleep before the scheduled sleep opportunity, the participants were constantly supervised and engaged in selected table games. At their habitual bedtime, i.e., 4.5 hours before a 4-hour sleep opportunity scheduled according to the sleep-wake habits of each participant during the two weeks prior to the study, we administered a time-controlled, pulsatile-release formula of caffeine or placebo (matched in appearance). The study followed a randomized, double-blind, placebo-controlled, cross-over design.

The pulsatile-release caffeine formula was previously described in detail (Dornbierer et al., 2021). In brief, 160 mg caffeine per capsule was dispersed in coating media and sprayed onto inert microcrystalline cellulose spheres, to obtain various layers consisting of caffeine and release-controlling polymers. The micropellets were then encapsulated into hydroxypropyl-methylcellulose capsules.

### **Caffeine quantification**

To monitor the caffeine pharmacokinetics during polysomnographically recorded sleep in the soundproof and climatized bedrooms of the human sleep research unit, we collected blood samples from the left antecubital vein at baseline (0.5 hours before capsule intake) and 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 13.5 and 17.5 hours after capsule administration. The venous catheter was connected to a blood-collection setup in an adjacent room (Heidelberger plastic tube extensions through the wall) (Dornbierer et al., 2021). Blood samples (4 ml, BD Vacutainer EDTA) were collected without disturbing the sleeping study participants. The intravenous line was kept patent with a slow drip (10 ml/h) of heparinized saline (1000 IU heparin in 0.9 g NaCl/dl; HEPARIN Bichsel; Bichsel AG, 3800 Unterseen, Switzerland). Blood samples were immediately centrifuged

for 10 min at 2000 relative centrifugal force (RCF) and plasma samples were immediately put on ice until final storage at  $-80^{\circ}\text{C}$ .

For the quantification of caffeine in plasma, we used an ultra-high performance liquid chromatography system coupled to a linear ion trap quadrupole mass spectrometer operated in positive electrospray ionization with scheduled multiple reaction monitoring. We previously reported all experimental and analytical procedures in detail (Dornbierer et al., 2021). Because of unreliable measurements, we needed to exclude the caffeine concentration data of one participant from the analyses (Dornbierer et al., 2021).

To estimate the caffeine concentration at timepoints of interest during sleep, we fitted the hourly data collected after capsule intake using polynomial regression ( $R^2 = 0.936$ ; R package stats v. 4.2.1). Subsequently the regression curve was used to interpolate caffeine concentration during all sleep stages and epochs.

### **Polysomnography**

We recorded sleep with dedicated polysomnographic amplifiers (Artisan®, Micromed, Mogliano, Veneto, Italy). The recording setup consisted of 10 EEG electrodes according to the 10–20 system (Fp1, Fp2, F3, F4, C3, C4, P3, P4, O1, O2), a bipolar electrooculogram (EOG), a submental electromyogram (EMG), and a 2-lead electrocardiogram (ECG). We marked the individual EEG electrode coordinates by cutting a few hairs at the electrode positions, to ensure that the electrodes were placed at the same place in both experimental conditions. We sampled the analogue signals with a frequency of 256 Hz, conditioned them by a high-pass filter (EEG: -3 dB at 0.15 Hz; EMG: 10 Hz; ECG: 1 Hz) and an antialiasing low-pass filter (-3 dB at 67.2 Hz), and stored the digitized data with a resolution of 256 Hz. Because of insufficient quality, we needed to exclude the EEG data of one participant from the analyses.

We analyzed the polysomnographic data in Rembrandt® Datalab (Version 8; Embla Systems, Planegg, Germany). We visually scored waking and sleep stages in 30-s epochs according to the criteria of the American Academy of Sleep Medicine (Berry et al., 2017). We visually identified and excluded movement- and arousal-related artifacts from the analyses.

We identified NREM-REM sleep cycles according to the criteria proposed by Feinberg & Floyd (Feinberg & Floyd, 1979) with the recently published R-package SleepCycles (v.1.1.4) (Blume & Cajochen, 2021). We visually inspected the results and corrected them if necessary. All participants completed at least two NREM-REM sleep cycles during the 4-hour sleep opportunity. We used the R-package bspec (version 1.6) to compute the EEG power spectra between 0 and 30 Hz derived from the C3-A2 derivation by a Fast Fourier Transform (FFT) based on 4-s epochs (Hanning window, linear detrending, 50% overlap), resulting in a frequency resolution of 0.25 Hz. We averaged spectral power in wakefulness (W), NREM sleep (stages N1, N2 and N3) and REM sleep. To investigate the evolution of EEG delta activity across the first two NREM–REM sleep cycles, we subdivided individual NREM sleep episodes into 20 equal parts and individual REM sleep episodes into four equal parts, and then averaged across all individuals (Landolt et al., 1995).

## Heart rate analyses

We identified the R-peaks in the ECG signal using the R-package `rsleep` (version 1.0.4) and the `scipy python` package (version 1.10.0), manually confirmed the results and corrected them if necessary. Using the R-package `RHRV` (version 4.2.6), we computed HRV measures in the frequency domain in all sleep states during the first two NREM–REM sleep cycles. With the FFT algorithm implemented in the `RHRV` package, we quantified the power spectral density of HRV in the following frequency bands: high-frequency (HF; 0.15–0.4 Hz); low-frequency (LF; 0.05–0.15 Hz); very-low-frequency (VLF; 0.03–0.05 Hz); ultra-low-frequency (ULF; < 0.03 Hz). Because the physiological underpinnings of the low-frequency components in the HRV spectrum are complex and partly uncertain, and because we were particularly interested in the effects of caffeine on parasympathetic activity, which is reliably reflected by HF-HRV (Berntson et al., 1997), we restricted the analyses to HF-HRV.

## Statistical analyses

We based all analyses on the complete data set of 20 participants using R version 4.2.1 (R Core Team, 2018) and RStudio Version 2022.07.1-554 (RStudio, Inc.). We analyzed the data with linear mixed effects models (R-package `lme4` v.1.1-30 and `lmerTest` v. 3.1-3) or two-sided, paired t-tests where specified (R package `rstatix` v.0.7.0). We included the fixed effects ‘cycle’ (1, 2), ‘state’ (W, N1, N2, N3, REM) and/or ‘condition’ (placebo, caffeine), as well as their interactions in the statistical models. When multiple measurements per subject were available, we added ‘study participant’ as random effect. We checked distribution of residuals, goodness of fit and assumptions in all tests and models. In all Figures, we present group means and 95% confidence intervals, based on 1000 bootstrap replicates (R-package `boot` v.1.3-28) (Efron & Tibshirani, 1993). To compare the placebo and caffeine conditions when the ANOVA ‘condition’ term yielded a significant result, we computed general linear hypothesis tests (R-package `multcomp` v. 1.4-19), corrected for multiple comparison with the Benjamini-Hochberg procedure. We also computed Cohen’s *d* measures, to quantify the effect size of the statistical differences (R-package `rstatix` v. 0.7.0) (Cohen, 1988).

We used generalized additive models, to analyze the change in EEG delta activity, heart rate and HF-HRV as non-linear function of the caffeine concentration (R-package `mgcv` v.1.8-40, `splines` v.4.2.1). We computed the range of significance by comparing the temporal evolution of the fitted smooth function in the caffeine and placebo conditions (R-package `mgViz` v.0.1.9, `gratia` v.0.7.3). All graphics were generated using R-packages `ggplot2` (v.3.3.6) or `ggpubr` (v.0.4.0).

### 3.4 Results

#### Caffeine plasma concentration

The delayed, pulsatile-release of caffeine started shortly before the sleep opportunity, and the caffeine in plasma exhibited a mean maximal concentration of  $9.60 \pm 0.89 \mu\text{mol/l}$  ( $\pm$  SEM,  $n=19$ ) 9.5 hours after capsule intake (Fig. 12A). At lights-off, i.e., at the beginning of the sleep opportunity, the mean caffeine concentration equaled  $4.19 \pm 0.82 \mu\text{mol/l}$ , with individual values varying between  $0.14\text{-}10.20 \mu\text{mol/l}$ . At lights-on of scheduled sleep, the mean caffeine concentration equaled  $8.00 \pm 0.86 \mu\text{mol/l}$ , varying between  $0.65\text{-}16.4 \mu\text{mol/l}$  (Fig. 1B). The increase in caffeine across the 4 hour sleep opportunity varied between  $0.51\text{-}16.9 \mu\text{mol/l}$  per individual.

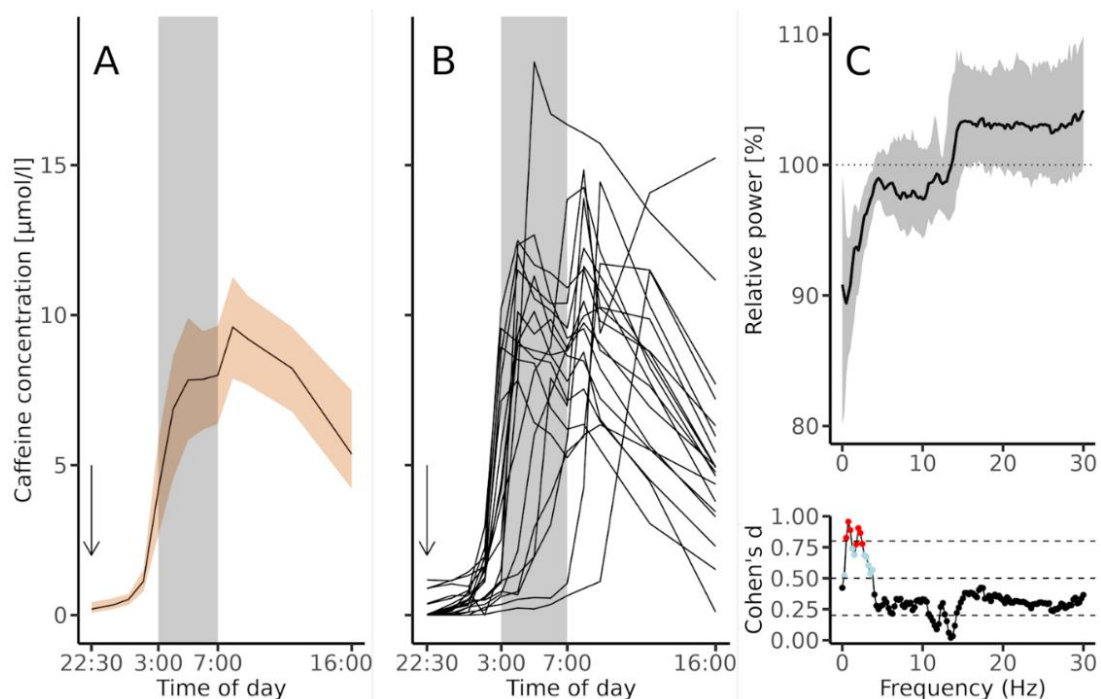


Figure 12: (A) Temporal evolution of the mean caffeine concentration in plasma (mean  $\pm$  95% confidence interval;  $n = 19$ ). The x-axis refers to clock time. The vertical arrow indicates the time when the time-controlled, pulsatile-release formula containing 160 mg caffeine was ingested. Grey shading indicates the time-in-bed for sleep (B) Temporal evolution of individual caffeine kinetics of the 19 participants for whom caffeine data were available. (C) Relative EEG power spectra in NREM sleep (stages N1-N3). For each 0.25-Hz frequency bin between 0-30 Hz, mean power ( $\pm$  95% confidence interval;  $n = 21$ ) in the caffeine condition was expressed as a percentage of the corresponding value in the placebo condition (horizontal dashed lines at 100%). Bottom panel: Effect size of the difference between caffeine and placebo expressed as Cohen's  $d$ . Red dots:  $p_{FDR} < 0.05$ ; grey dots:  $p_{uncorrected} < 0.05$ ; black dots:  $p_{uncorrected} > 0.05$ .

## Effects of caffeine on the EEG during sleep

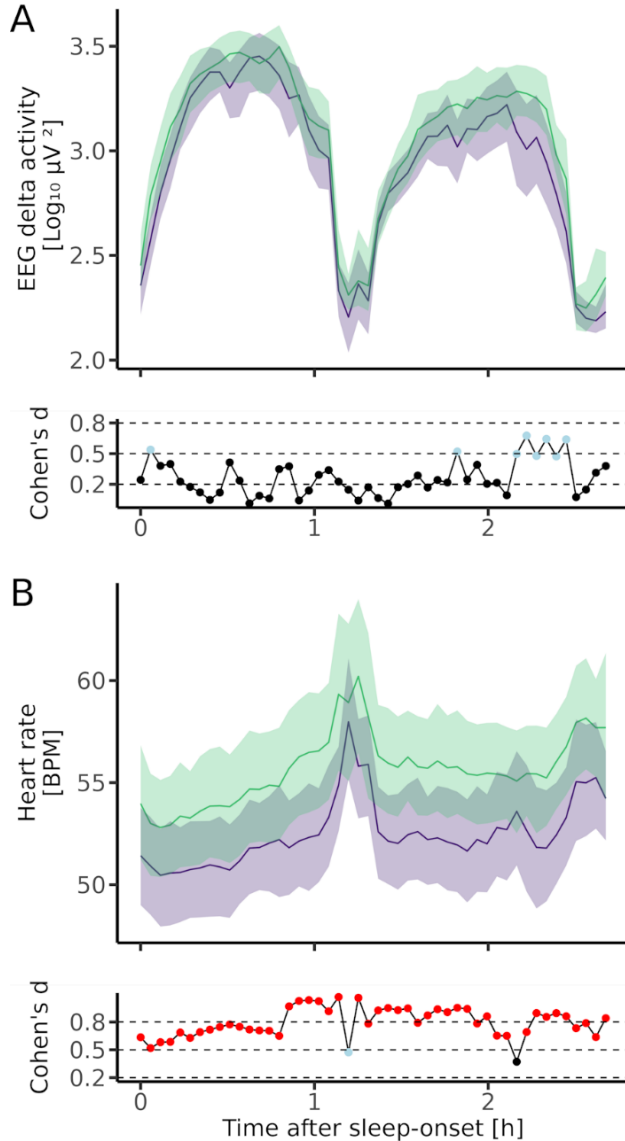


Figure 13: (A) Time course of EEG delta activity (0.75-2.5 Hz) across the first two NREM-REM sleep episodes in the placebo (green) and caffeine (purple) conditions (mean  $\pm$  95% confidence intervals;  $n = 21$ ). Individual NREM sleep episodes were subdivided into 20 and individual REM sleep episodes into 4 equal time bins. The data were aligned with respect to sleep onset, averaged per condition, and plotted against the mean timing of all NREM and REM sleep episodes. Bottom panel: Effect size of the difference between caffeine and placebo expressed as Cohen's  $d$ . Grey dots:  $p_{\text{uncorrected}} < 0.05$ ; black dots:  $p_{\text{uncorrected}} > 0.05$ . (B) Time course of heart rate across the first two NREM-REM sleep episodes in the placebo (green) and caffeine (purple) conditions (mean  $\pm$  95% confidence intervals;  $n = 21$ ). Individual NREM sleep episodes were subdivided into 20 and individual REM sleep episodes into 4 equal time bins. The data were aligned with respect to sleep onset, averaged per condition, and plotted against the mean timing of all NREM and REM sleep episodes. Bottom panel: Effect size of the difference between caffeine and placebo expressed as Cohen's  $d$ . Red dots:  $p_{\text{FDR}} < 0.05$ ; grey dots:  $p_{\text{uncorrected}} < 0.05$ ; black dots:  $p_{\text{uncorrected}} > 0.05$ .

The visually scored sleep variables in the first two NREM-REM sleep cycles in the placebo and caffeine conditions are summarized in supplementary Table S1. In both conditions, the participants quickly fell asleep and exhibited a high proportion of slow wave sleep. Except for the concentration-dependent, reduced time spent in N3 sleep after caffeine compared to placebo ( $69.7 \pm 19.6$  vs.  $55.8 \pm 19.5$ ,  $p_{\text{FDR}} < 0.03$ ) (also see supplementary Fig. S1), sleep architecture in both experimental conditions was comparable ( $p_{\text{all}} > 0.1$ ). Corroborating less visually scored N3 sleep, caffeine reduced EEG delta activity in NREM

sleep. While power in all bins between 0.5-3.75 Hz was reduced, correction for multiple comparisons revealed that the reduction in the 0.75-2.5 Hz range was statistically significant (Fig. 12C). The caffeine intake reduced EEG activity in this frequency band with a large effect size when compared to placebo (average Cohen's  $d$   $0.86 \pm 0.02$  [SEM]).

The evolution of EEG power in the 0.75-2.5 Hz band in the placebo and caffeine conditions across the first two NREM-REM sleep cycles is illustrated in Fig. 13A. Delta activity varied across wakefulness and sleep states ('state':  $F_{4,180} = 164.18$ ,  $p < 0.0001$ ). It decreased from the 1st to the 2nd NREM sleep episode ('cycle':  $F_{1,60} = 20.32$ ,  $p < 0.0001$ ) and was reduced after caffeine when compared to placebo ('condition':  $F_{1,60} = 7.52$ ,  $p < 0.01$ ). The mean effect size of the difference between the conditions equaled  $0.28 \pm 0.03$  (SEM), indicating a small to medium effect. A decrease in 0.75-2.5 Hz activity was also present from the 1st to the 2nd REM sleep episode

(‘cycle’:  $F_{1,37.2} = 6.66$ ,  $p < 0.02$ ), whereas the caffeine and placebo conditions did not differ (‘condition’:  $F_{1,36.2} = 3.83$ ,  $p > 0.058$ ).

### Concentration-effect relationship between caffeine and reduction in NREM sleep delta activity

To investigate whether the effect of caffeine on delta activity was concentration dependent, the difference between caffeine and placebo in 0.75-2.5 Hz activity in all time bins during the first two NREM sleep episodes was expressed as a function of the caffeine concentration. Computing the linear correlation coefficient between the mean difference in delta activity and the mean caffeine concentration in the first two NREM sleep episodes revealed a negative association ( $r_{\text{Pearson}} = -0.62$ ,  $p < 0.005$ ). The generalized-additive, non-linear model function revealed a significant distance from the no-effects line for values above  $7.35 \mu\text{mol/l}$ , suggesting a significant impact of caffeine on EEG delta activity in NREM sleep above this value (Fig. 14).

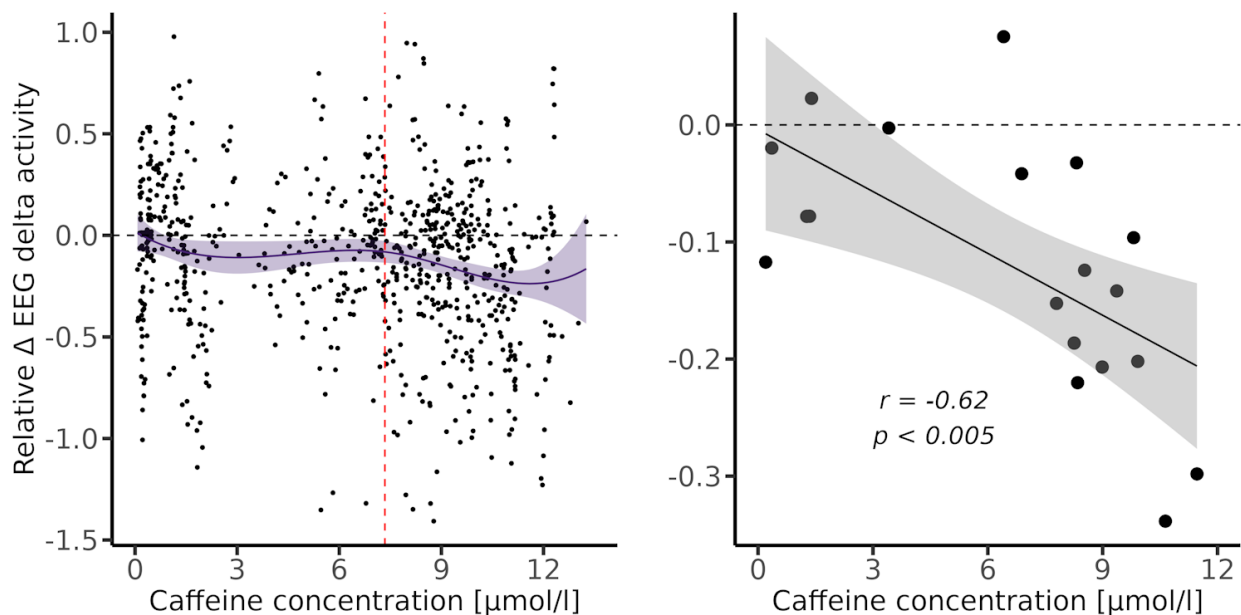


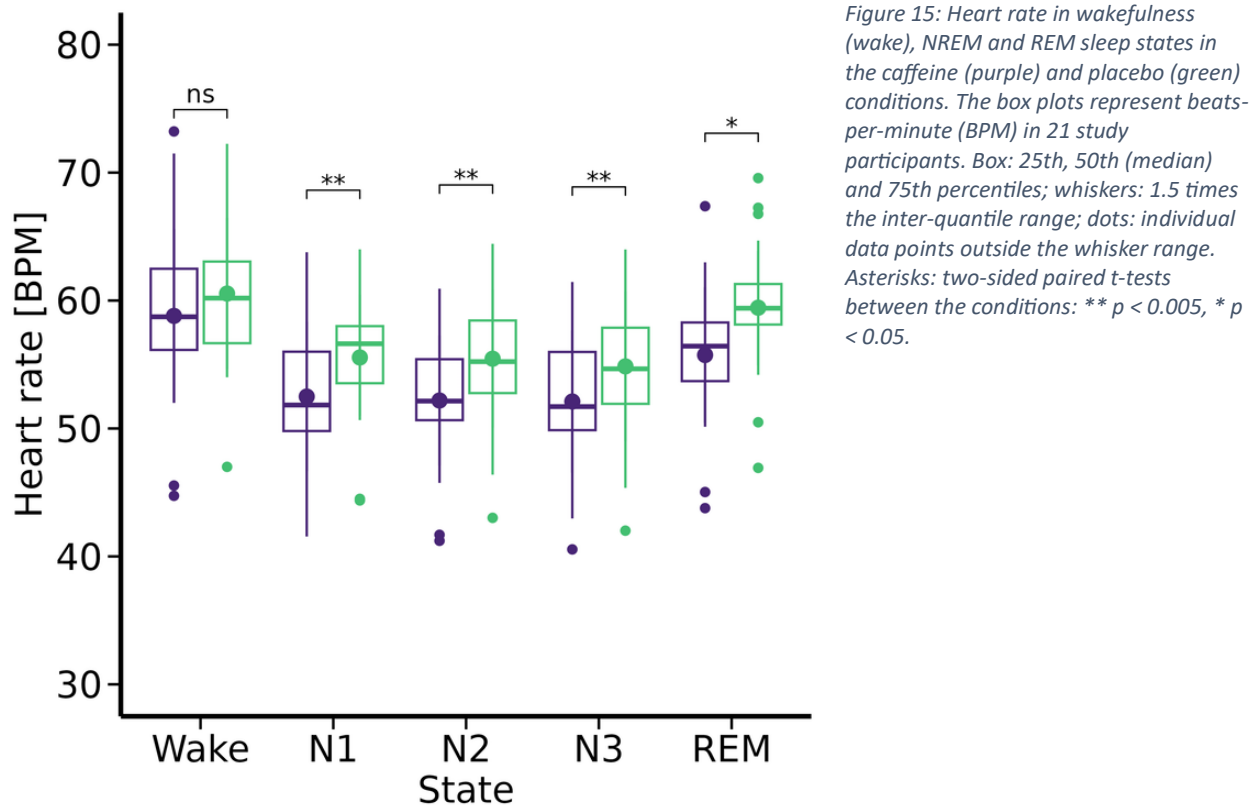
Figure 14: Left panel: Difference in EEG delta activity (0.75-2.5 Hz) between the caffeine and placebo (horizontal dashed line) conditions in  $2 \times 20$  equal time bins of the NREM sleep episodes 1 and 2. Individual data points in 19 participants and their averages (black line  $\pm$  95% confidence intervals) are plotted. The red vertical dashed line at  $7.34 \mu\text{mol/l}$  marks the threshold above which the average difference between caffeine and placebo was estimated to be higher than three times the standard error of the null hypothesis. Right panel: Pearson product moment correlation between the difference in EEG delta activity (0.75-2.5 Hz) between caffeine and placebo and the mean caffeine in plasma in the first two NREM sleep episodes ( $n = 19$ ). The continuous black line shows the corresponding linear trend.

### Effects of caffeine on heart rate during sleep

The time course of heart rate in the placebo and caffeine conditions across the first two NREM-REM sleep cycles is illustrated in Fig. 2B. After a small drop immediately upon sleep onset, heart rate remained low in NREM sleep and transiently increased in REM sleep (‘state’, NREM vs. REM sleep:  $F_{1,60} = 58.79$ ,  $p < 0.0001$ ). Heart rate slightly increased from the 1st to the 2nd NREM sleep

episode ( $F_{1,60} = 4.11$ ,  $p < 0.05$ ), while it did not differ between the episodes in REM sleep ( $F_{1,34.3} = 0.17$ ,  $p = 0.68$ ).

The modulation of heart rate by the different states of vigilance and caffeine is illustrated in Fig. 4. Mean heart rate differed among wakefulness and sleep ('state':  $F_{4,180} = 47.12$ ,  $p < 0.0001$ ), with the lowest values in NREM sleep. Compared to placebo, caffeine induced an overall reduction in heart rate by  $3.24 \pm 0.77$  (SEM) beats-per-minute (BPM) ( $F_{1,180} = 64.79$ ,  $p < 0.0001$ ). This difference corresponded to a large effect size (Cohen's  $d = 0.79 \pm 0.02$ ). In NREM sleep, caffeine reduced heart rate by  $3.19 \pm 0.78$  BPM ( $F_{1,60} = 36.51$ ,  $p < 0.0001$ ; N1:  $t_{20} = 4.03$ ; N2:  $t_{20} = 4.42$ ; N3:  $t_{20} = 4.21$ ;  $p_{\text{all}} = 0.001$ ), which corresponded to a large effect size (Cohen's  $d = 0.79 \pm 0.03$ ). The reduction in REM sleep equaled  $3.72 \pm 1.01$  BPM ( $F_{1,34.3} = 22.16$ ,  $p < 0.0001$ ;  $t_{20} = 2.73$ ,  $p = 0.012$ ), also corresponding a large effect size (Cohen's  $d = 0.8 \pm 0.07$ ). In wakefulness, the difference between the conditions was not significant ( $t_{20} = 1.67$ ,  $p > 0.1$ ).



### Concentration-effect relationship between caffeine and reduction in heart rate in NREM sleep

Correlation analysis of the averaged values per participant (i.e., the average of the 2 x 20 time-bin values in NREM sleep) showed a negative correlation between the change of heart rate and the caffeine concentration ( $r_{\text{Pearson}} = -0.66$ ,  $p < 0.003$ ). Generalized additive model estimates revealed a significant distance from the null-effects line (no difference) above caffeine amounts of  $4.25 \mu\text{mol/l}$ , suggesting that a caffeine concentration above this value reduces heart rate (Fig. 16A).

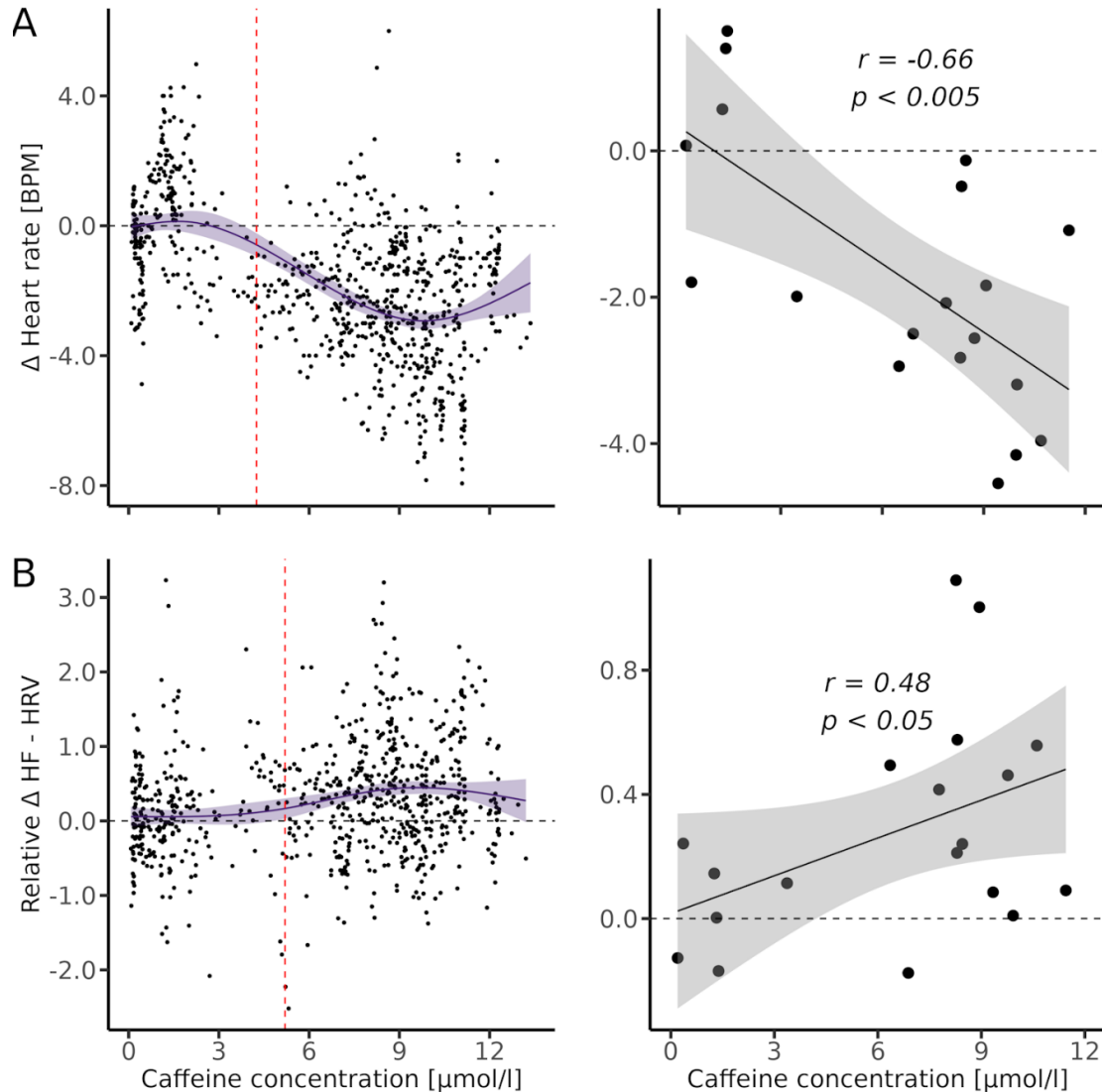


Figure 16: (A) Left panel: Difference in heart rate (beats-per-minute, BPM) between the caffeine and placebo (horizontal dashed line) conditions in 2 x 20 equal time bins of the NREM sleep episodes 1 and 2. Individual data points in 19 participants and their averages (black line  $\pm$  95% confidence intervals) are plotted. The red vertical dashed line at 4.25  $\mu\text{mol/l}$  marks the threshold above which the average difference between caffeine and placebo was estimated to be higher than three times the standard error of the null hypothesis. Right panel: Pearson product moment correlation between the difference in heart rate between caffeine and placebo and the mean caffeine in plasma in the first two NREM sleep episodes ( $n = 19$ ). The continuous black line shows the corresponding linear trend. (B) Same representation of high-frequency (0.15-0.4 Hz) spectral power in heart rate variability (HR-HRV) as in panel A. The red vertical dashed line at 4.92  $\mu\text{mol/l}$  marks the threshold above which the average difference between caffeine and placebo was estimated to be higher than three times the standard error of the null hypothesis.

### Concentration-effect relationship between caffeine and heart rate variability in NREM sleep

To estimate caffeine-related changes of parasympathetic activity during sleep, we quantified HF-HRV. Caffeine increased the HF-HRV component in NREM sleep by  $0.23 \pm 0.08 \log \text{ms}^2$  ( $F_{1,20} = 7.85$ ,  $p < 0.05$ ,  $t_{20} = 2.80$ ,  $p < 0.05$ , Cohen's  $d = 0.25$ ). Mean individual changes in HF-HRV in the first two NREM sleep episodes correlated positively with the increasing plasma caffeine

concentration ( $r_{\text{Pearson}} = 0.48$ ,  $p < 0.05$ ). The non-linear analysis of the caffeine related change in HF-HRV showed a deviation from the null-effects baseline for caffeine concentrations exceeding  $4.92 \mu\text{mol/l}$  (Fig. 16B).

### 3.5 Discussion

In this study, we systematically investigated the concentration-effect relationships among the caffeine levels in plasma, the sleep EEG, heart rate, and HF-HRV during human sleep. Using powerful, generalized additive models to probe non-linear associations among these variables, we found concentration-dependent alterations in EEG 0.75-2.5 Hz activity above  $\sim 7.3 \mu\text{mol/l}$  and in heart rate and HF-HRV above  $\sim 4\text{-}5 \mu\text{mol/l}$  plasma caffeine. The effects were generally characterized by a consistent and large effect size. The findings suggest that above a certain threshold, caffeine reduces EEG delta activity and increases cardiac autonomic activity in NREM sleep in a concentration-dependent manner. The exact modes of action how caffeine affects these central and autonomic nervous system functions are complex and remain to be fully elucidated. Sleep architecture, the sleep EEG and cardiac autonomic activity are highly sensitive to subtle internal and external influences such as moderate doses of caffeine. When previous studies aimed at elucidating the concentration- and exposure-effect relationships between caffeine pharmacokinetics and sleep physiologic measures, they typically relied on the caffeine content in a single saliva or blood sample before sleep initiation. Nevertheless, uptake, metabolism and excretion of exogenous caffeine are dynamic processes, which differ widely among individuals. The simultaneous quantification of pharmacokinetic and physiological variables is necessary to understand how they are mutually related. By doing so and capitalizing from the comprehensive characterization of a newly developed, time-controlled, pulsatile-release caffeine formula (Dornbierer et al., 2021), we observed that a caffeine plasma level above  $7.3 \mu\text{mol/l}$  is required to reduce EEG 0.75-2.5 Hz activity in NREM sleep. Above this concentration, we found a robust negative association between this physiological marker of NREM sleep intensity and the level of caffeine.

In humans, the actions of caffeine on the central nervous system are more often quantified by behavioral changes than by direct changes of brain electrical activity. Early experimental work concluded that intake of 80-100 mg caffeine shortly before bedtime is required, to delay the onset of sleep (reviewed by Dews 1982). Furthermore, based on pharmacokinetic estimates, it was suggested that a plasma concentration of  $\sim 7 \mu\text{mol/l}$  ( $> 2.5 \mu\text{g/ml}$ ) is the threshold for the effective promotion of vigilance during prolonged wakefulness (Beaumont et al., 2001).

Interestingly, the present data indicate that a plasma caffeine concentration in this range ( $\sim 7.3 \mu\text{mol/l}$ ) is necessary to attenuate EEG delta activity in NREM sleep. Given that the saliva/plasma concentration ratio is stable at  $\sim 0.74$  (Newton et al., 1981),  $7 \mu\text{mol/l}$  caffeine in plasma would correspond to  $\sim 10 \mu\text{mol/l}$  caffeine in saliva. Caffeine intake in the morning and during prolonged wakefulness was repeatedly found to attenuate subsequent EEG slow-wave activity during sleep at concentrations far below this threshold (Landolt et al., 1995; Landolt et al., 2004; Rétey et al., 2007). As discussed in more detail elsewhere, these caffeine-induced changes could reflect the attenuated build-up of homeostatic sleep pressure during wakefulness or the continued

antagonism of adenosine receptors by active caffeine metabolites such as paraxanthine (Reichert et al., 2022b).

Consistent with previous studies in wakefulness, we found that moderate caffeine decreased heart rate and increased HRV, and that caffeine affects these measures of cardiac autonomic activity in non-linear fashion (Crooks et al., 2019; Kohler et al., 2006; for systematic review, see Koenig et al., 2013). Non-linear statistical analyses revealed that above a plasma concentration of  $\sim 4.3 \mu\text{mol/l}$ , caffeine reduced heart rate on average by more than 3 bpm and that the decrease correlated with increased caffeine levels. Compared to placebo, the heart rate was reduced to a similar extent in all sleep states and wakefulness (we found no significant 'state' x 'condition' interaction). It was previously hypothesized that low and high doses of caffeine independently affect cardiovascular variables via different underlying mechanisms (Fredholm et al., 2017). While the effects of low-dose caffeine such as in this study may be mediated by adenosine receptor blockade, caffeine at higher doses may induce increased intracellular calcium, norepinephrine release or sensitization of dopamine receptors (Fredholm et al., 2017; Temple et al., 2017). The present research does not allow to elucidate the physiological mechanism underpinning the reduced heart rate. We speculate that it could reflect a baroreflex-induced bradycardia caused by caffeine-induced rise in blood pressure (de Zambotti et al., 2018). Baroreflexes regulate blood pressure, heart rate, and blood volume within a narrow range by activating baroreceptors located in major arteries, veins, and the heart. These stretch-activated receptors signal to the nucleus of the solitary tract of the brain stem through the vagus and glossopharyngeal nerves. They evoke reflex inhibition of sympathetic signals to blood vessels, causing vasodilatation, and increase parasympathetic nerve activity to the sinoatrial node, slowing heart rate (Kaufmann et al., 2020). The exact response of the baroreflex and the autonomic nervous system can vary depending on the sleep stage, stress level and overall health status (Penzel et al., 2016).

To estimate vagally-mediated, parasympathetic nervous system activity, we quantified the HF-HRV during sleep (de Zambotti et al., 2018; Koenig et al., 2013). Compared to placebo, we found that this measure was increased for caffeine levels above  $\sim 4.9 \mu\text{mol/l}$ , and the increase correlated with increased caffeine levels. Assuming normal sinus rhythm and atrioventricular functions, increased HF-HRV may reflect the parasympathetic modulation of normal R-R intervals driven by ventilation (Kleiger et al., 2005).

Because we did not quantify the evolution of blood pressure and breathing patterns during sleep, future studies are necessary to investigate this hypothesis. In addition, we don't know whether the caffeine metabolites paraxanthine, theophylline and theobromine contributed to the observed effect. The metabolic breakdown of caffeine underlies high inter-individual variation and each of the major metabolites affect adenosine receptors in body organs with different affinity and via different downstream mechanisms. These unknown secondary effects of caffeine may contribute to the margin of error, which is evident in the heterogeneous distribution of some data reported.

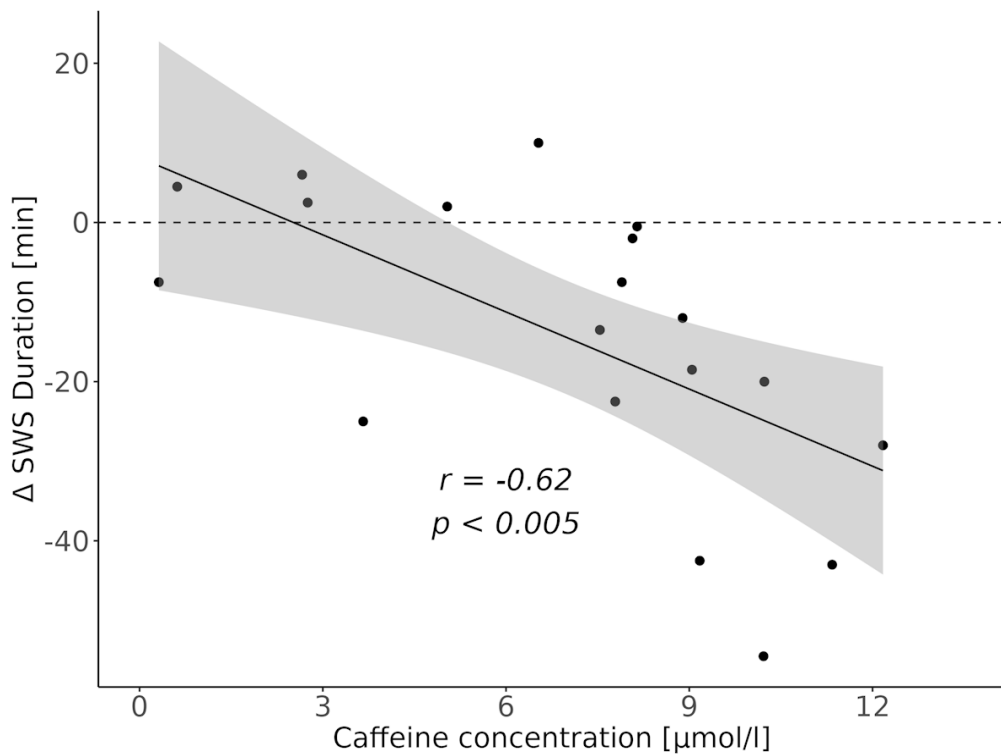
### 3.6 Supplementary information

**Supplementary Table 9.** Visually scored sleep variables during the first two NREM-REM sleep cycles.

Variable	Placebo		Caffeine		t-value	df	p <sub>FDR</sub>
	Mean	SEM	Mean	SEM			
TST	153.78	5.79	142.84	6.71	-1.70	20	0.2297
SL	11.93	4.38	13.81	3.63	0.82	20	0.5500
RL	70.26	11.08	87.67	11.58	1.11	20	0.4030
N1	4.95	0.84	7.62	0.92	2.57	20	0.1014
N2	56.19	3.84	55.07	2.71	-0.30	20	0.7660
N3	69.67	4.27	55.76	4.26	-3.57	20	<b>0.0250</b>
REM sleep	18.81	4.00	20.64	3.44	0.42	20	0.7334
WASO	5.78	2.00	10.9	3.83	1.27	20	0.3575

Mean values are reported in minutes ( $\pm$  the standard error of the mean [SEM]) for 21 study participants. Total sleep time (TST): time spent in sleep stages N1, N2, N3 and REM sleep between sleep onset and the end of the second REM sleep episode. Sleep latency (SL): time between lights-off and first occurrence of stage N2. REM sleep latency (RL): time between lights-off and first occurrence of REM sleep. N1, N2, N3: non-rapid-eye-movement sleep stages. REM sleep: rapid-eye-movement sleep. WASO: wakefulness after sleep onset. Paired t-test was used for the comparisons between the conditions; false discovery rate (FDR) was used to correct for multiple comparison.

**Supplementary Figure 3.**



Pearson product moment correlation between the difference in slow wave sleep (min) between the caffeine and placebo conditions, and the mean caffeine concentration in blood plasma in the first two NREM sleep episodes. The continuous black line illustrates the corresponding linear trend ( $r = -0.62$ ,  $p < 0.005$ ,  $n = 19$ ).

## 4 A novel bedtime pulsatile-release caffeine formula ameliorates sleep inertia symptoms immediately upon awakening

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## 4.1 Abstract

Sleep inertia is a disabling state of grogginess and impaired vigilance immediately upon awakening.

The adenosine receptor antagonist, caffeine, is widely used to reduce sleep inertia symptoms, yet the initial, most severe impairments are hardly alleviated by post-awakening caffeine intake. To ameliorate this disabling state more potently, we developed an innovative, delayed, pulsatile-release caffeine formulation targeting an efficacious dose briefly before planned awakening. We comprehensively tested this formulation in two separate studies. First, we established the *in vivo* caffeine release profile in 10 young men. Subsequently, we investigated in placebo-controlled, double-blind, cross-over fashion the formulation's ability to improve sleep inertia in 22 sleep-restricted volunteers. Following oral administration of 160 mg caffeine at 22:30, we kept volunteers awake until 03:00, to increase sleep inertia symptoms upon scheduled awakening at 07:00. Immediately upon awakening, we quantified subjective state, psychomotor vigilance, cognitive performance, and followed the evolution of the cortisol awakening response. We also recorded standard polysomnography during nocturnal sleep and a 1h nap opportunity at 08:00. Compared to placebo, the engineered caffeine formula accelerated the reaction time on the psychomotor vigilance task, increased positive and reduced negative affect scores, improved sleep inertia ratings, prolonged the cortisol awakening response, and delayed nap sleep latency one hour after scheduled awakening. Based on these findings, we conclude that this novel, pulsatile-release caffeine formulation facilitates the sleep-to-wake transition in sleep-restricted healthy adults. We propose that individuals suffering from disabling sleep inertia may benefit from this innovative approach.

## 4.2 Introduction

Sleep inertia is a disabling state of reduced physical and mental drive following awakening (Hilditch & McHill, 2019), which typically lasts for less than 30 min but symptoms may persist for several hours in susceptible individuals (Hofer-Tinguely et al., 2005; Jewett et al., 1999; Tassi & Muzet, 2000). A large portion of healthy adolescents report persistent difficulties to rise in the morning (Amaral et al., 2014) and many shift and on-call night workers exhibit impaired performance and grogginess after awakening, which raises important safety concerns in operational settings (Hilditch et al., 2016; Ohayon et al., 2000). Furthermore, impaired post-awakening vigilance and mood is highly prevalent in a broad range of neurological and psychiatric conditions (Borisenkov et al., 2015; Ohayon et al., 2000; Trenkwalder et al., 2011; Trotti & Bliwise, 2014).

Several biological and environmental factors influence the manifestation of sleep inertia. For example, abrupt awakening from deep sleep (also referred to as slow-wave sleep or stage N3 of non-rapid-eye-movement [NREM] sleep) is associated with more severe sleep inertia symptoms when compared to awakening from more superficial NREM sleep and rapid-eye-movement (REM) sleep (Broughton, 1968; Bruck & Pisani, 1999). This finding may suggest that neurophysiological processes underlying sleep and particularly deep NREM sleep carry over into wakefulness and contribute to behavioral and cognitive deficits associated with sleep inertia

(Vyazovskiy et al., 2014). The proportion of deep sleep and the odds of awakening from N3 sleep is increased when a sleep opportunity is shorted such as in sleep restriction. Accordingly, abrupt awakening during sleep restriction enhances sleep inertia symptoms (Tassi & Muzet, 2000).

The neuromodulator adenosine is a key regulator of deep sleep (Landolt, 2008) and adenosinergic neuromodulation may play an essential role in the manifestation of sleep inertia (Nehlig, 1999). Consistent with this view, the adenosine receptor antagonist, caffeine, is widely used to counteract sleep inertia. Besides attenuating sleepiness and deficits in vigilance (Rupp et al., 2013; Van Dongen et al., 2001), caffeine augments cardiovascular and respiratory functions (Gonzaga et al., 2017) and promotes the release of cortisol, a key hormone of the hypothalamic–pituitary–adrenal (HPA) axis (Lin et al., 1997). The HPA axis regulates several psycho-vegetative aspects of the wake-up process, including the cortisol awakening response (CAR). The CAR reflects HPA axis function (Boehringer et al., 2015; Clow et al., 2010) and may be associated with the propensity of sleep inertia (Kudielka et al., 2006).

Because the impairments are most severe immediately upon awakening, proactive strategies aiming for optimal sleep length and timing have been recommended to minimize sleep inertia symptoms (Hilditch et al., 2016). Nevertheless, it is not always possible to plan and obtain sleep of sufficient length and quality and at the optimal time of day. On the other hand, currently there exists no convincing evidence that reactive countermeasures to sleep inertia, i.e. strategies implemented upon wake-up, are sufficiently effective (Hilditch et al., 2016). Although caffeine is the best available option, coffee takes 20–30 min to have an alerting effect (Bonati et al., 1982). The bioavailability of reactive oral caffeine intake prevents effective amelioration of sleep inertia symptoms for at least 12–18 min upon waking (Newman et al., 2013), unless it is taken as a proactive countermeasure prior to a short sleep or nap bout (Centofanti et al., 2020; Van Dongen et al., 2001). When taken before sleep, however, caffeine can delay sleep onset, reduce total sleep time and attenuate the amount of deep slow wave sleep when a pharmacologically active concentration is present in the organism during sleep (Centofanti et al., 2020; Landolt, Dijk, et al., 1995).

The time-controlled administration of pharmaceuticals in accordance with the sleep–wake cycle provides an essential pillar in the emerging concept of chronopharmacology and chronotherapeutics (Ballesta et al., 2017; Mandal et al., 2010). We aimed at developing a chronotherapeutic caffeine formulation that ameliorates impaired subjective state, vigilance and performance immediately upon awakening without disturbing the quality of the preceding sleep episode. For this purpose, we invented a delayed, pulsatile-release caffeine delivery system targeted to reach an efficacious plasma concentration approximately 7h after intake. When ingested at habitual bedtime, we hypothesized that this formula would improve vigilance and mood, elevate the CAR, and reduce sleep propensity on the subsequent morning after wake-up from nocturnal sleep. We tested these hypotheses in two separate studies. First, we examined the *in vivo* release properties of the engineered caffeine formula throughout a nocturnal sleep episode.

Then, we comprehensively investigated in randomized, double-blind, cross-over, placebo-controlled manner its effects on behavioral, emotional, neurocognitive and physiological symptoms of sleep inertia in sleep-restricted healthy young men.

## 4.3 Methods

### **Participants and permission**

A total of 32 healthy young men (mean age:  $25.6 \pm 3.7$  years) participated in the two studies (in vivo validation study:  $n = 10$ ; pharmacodynamic study:  $n = 22$ ), whereof 5 subjects participated in both experiments. The following criteria were required for inclusion: (i) male sex in order to avoid the potential impact of menstrual cycle on sleep physiology or HPA axis activity, (ii) age within the range of 18–34 years, (iii) a body-mass-index below 25, (iv) an Epworth Sleepiness Score (ESS) below 10, (v) habitual sleep onset latency below 20 min, (vi) regular sleep–wake rhythm with bedtime between 10 pm and 1 am, (vii) absence of any somatic or psychiatric disorders, (viii) no acute or chronic medication intake, (ix) non-smoking, (x) no history of drug abuse (lifetime use > 5 occasions, with exception of occasional cannabis use), and (xi) caffeine consumption of less than 4 units per day (coffee, tea, chocolate, cola, energy drinks).

The participants were instructed to abstain from illicit drugs and caffeine during the entire study, starting two weeks prior to the first experimental night until the end of the study (the day after the second experimental night).

No alcohol was allowed 24h before the experimental nights. The minimal wash out period before the experimental nights was 7 days. Participants were also instructed to keep an individual regular sleep-wake rhythm (23:00–07:00 or 22:00–06:00 depending on the volunteers' habitual bedtime) during the entire study, starting 2 weeks prior to the experimental night. All included participants chose to keep either a 22:00–06:00 rhythm or a 23:00–07:00 rhythm. To ensure adherence to the regular sleep–wake pattern, participants were instructed to wear a rest-activity monitor on the non-dominant arm and to keep a sleep–wake diary.

The studies were approved by the Cantonal Ethics Committee of the Canton of Zurich (BASEC: 2018-00533) and registered on ClinicalTrials.gov (Identifier: NCT04975360). All participants provided written informed consent according to the declaration of Helsinki.

### **Study drug**

The caffeine pulsatile-release formulation was manufactured using a drug layering process (Naeem et al., 2015). The details of the engineering and manufacturing processes will be reported elsewhere. In brief, caffeine and the excipients were dispersed in the coating media and then sprayed onto inert microcrystalline cellulose spheres using a fluid bed through a Wurster tube with continuous inlet air that dries the liquid in the dispersion, to obtain various layers consisting of caffeine and release-controlling polymers. The applied release-controlling polymeric system was based on methacrylate copolymers, which control the release of caffeine in both a pH-dependent and pH-independent manner (Akhgari et al., 2006; Song et al., 2019). Thereby, the release mechanism of the polymeric system was mainly driven by the swellability and permeability of the copolymers (Yadav et al., 2011). The final micropellets were then encapsulated into hydroxy-propyl-methylcellulose capsules.

To evaluate the in-vitro dissolution profiles of the manufactured formula, different prototypes were tested by means of state-of-the-art dissolution assays, mimicking gastrointestinal conditions (Broesder et al., 2020; Wahlgren et al., 2019). Development and in vitro testing of the caffeine pulsatile-release formulation and placebos was conducted at Elixir Pharmaceutical Research and

Development Corporation in Ankara. For the in vivo study, the most suited prototype with a favorable in vitro dissolution profile was chosen.

### In vivo validation study

In a first open-label evaluation study of the engineered delivery system, the in vivo caffeine release profile was determined in 10 fasted (no food or beverage consumption 2h before drug administration) male individuals. After oral intake at 22:30, study participants were allowed to sleep from 23:00 to 07:00, while blood was continuously sampled. Samples were collected from the left antecubital vein at baseline (22:00), and 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 13.5 and 17.5h after drug administration.

During the sleep episode in the soundproof and climatized bedrooms of the sleep laboratory, the venous catheter was connected to a blood-collection setup in an adjacent room (Heidelberger plastic tube extensions through the wall). Thus, blood samples (4ml, BD Vacutainer EDTA) were collected without disturbing the sleeping study participants. The intravenous line was kept patent with a slow drip (10 ml/h) of heparinized saline (1000 IU heparin in 0.9g NaCl/dl; HEPARIN Bichsel; Bichsel AG, 3800 Unterseen, Switzerland). Blood samples were immediately centrifuged for 10 min at 2000 RCF and plasma samples were immediately stored on ice until final storage at  $-80\text{ }^{\circ}\text{C}$ .

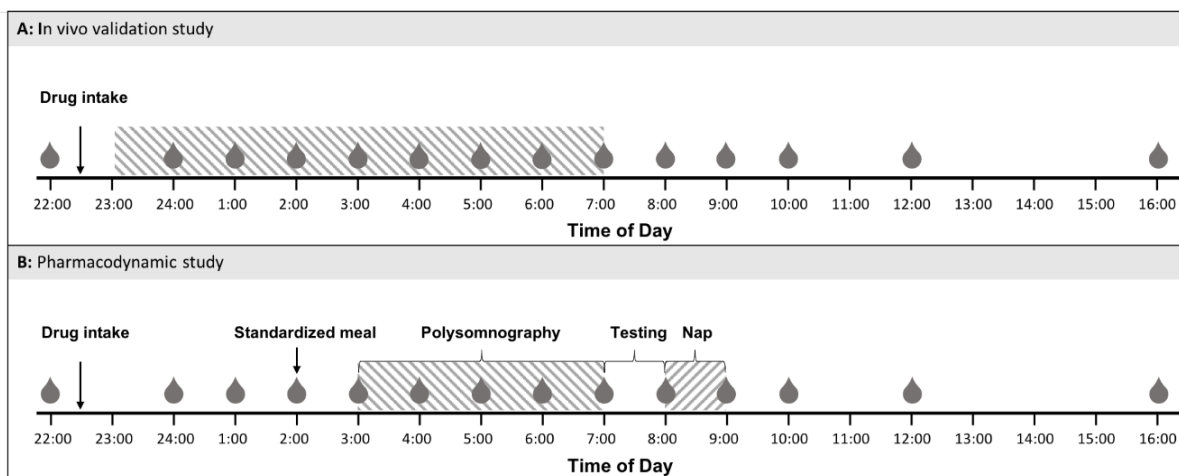


Figure 17: Illustration of the study procedures of the in vivo validation study (A) and the pharmacodynamic study (B). Timepoints of blood withdrawal are indicated as grey drops (drop symbols). Sleep episodes are highlighted as hatched areas. In both studies, sleep was continuously recorded by polysomnography. In the pharmacodynamic study, participants were kept awake until 3:00. Immediately after awakening from a restricted 4-h nocturnal sleep episode (at 07:00), volunteers performed a 1-h testing battery (referred to as “Testing”) to quantify behavioral, cognitive, emotional and physiological markers of sleep inertia. At 8:00, the participants were given a 1-h nap opportunity, while the latency to fall asleep and the sleep profile were recorded with polysomnography. Sleep inertia assessment, CAR measurements and physiological sleepiness testing were only performed in the pharmacodynamic study.

### Pharmacodynamic study

In the pharmacodynamic study, the pulsatile-release caffeine formulation (which was validated in vivo as described above) or a placebo (matched in appearance) were administered 8.5h before

the scheduled wake-up time to the fasted participants (no food or beverage consumption 2h before drug administration). To exacerbate sleep inertia symptoms and avoid neuropsychological ceiling/floor effects, which are frequent in intervention studies with highly functioning healthy volunteers, all participants were sleep restricted. More specifically, they were kept awake until 02:00 (participants adhering to a 22:00–06:00 rhythm) or 03:00 (23:00–07:00 rhythm), then given a 4-h sleep opportunity, and awoken at 06:00 (22:00–06:00 rhythm) or 07:00 (23:00–07:00 rhythm). At 3.5h post-administration, all volunteers received a standardized, light meal.

Blood was continuously sampled upon drug administration and the caffeine release from the formulation was monitored as described above. Upon awakening, the effects of the formulation on neurobehavioral, emotional, cognitive, and endocrinological markers of sleep inertia were assessed. Additionally, physiological sleep tendency was investigated by determining the sleep characteristics of a 1-h nap opportunity starting 1h post-awakening. To simplify descriptions and data presentations, we will only refer to the 23:00–07:00 rhythm with respect to the time-points of the tasks, because only a small minority of the participants followed the 22:00–06:00 rhythm.

The pharmacodynamic study followed a randomized, double-blind, placebo-controlled, crossover design with a wash-out period of at least 1 week between the caffeine and placebo conditions.

The details of both study designs are illustrated in Fig. 17.

### **Analysis of caffeine levels**

Caffeine and caffeine-<sup>13</sup>C<sub>3</sub> were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals used were of the highest purification grade available. Briefly, 200 µl of plasma, 50 µl of the internal standard (IS) (40 µM caffeine-<sup>13</sup>C<sub>3</sub>) and 50 µl of methanol (MeOH) were added to an Eppendorf tube.

For protein precipitation, 400 µl of acetonitrile (ACN) were slowly added. Samples were shaken for 10 min and centrifuged at 10,000 rpm for 5 min. A volume of 350 µl of the supernatant was transferred into an auto-sampler vial and evaporated to dryness under a gentle stream of nitrogen. For reconstitution, 250 µl of an eluent-mixture (95:5, v/v) was added. Quality control (QC) samples and calibrators (Cal) were prepared with the same sample preparation, replacing the 50 µl of MeOH with the Cal or QC solutions. The plasma samples were analyzed on an ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher, San Jose, CA, USA) coupled to a linear ion trap quadrupole mass spectrometer 5500 (Sciex, Darmstadt, Germany). The mobile phases of the

UHPLC consisted of water (eluent A) and a mixture (70:30 v/v) of MeOH and ACN (eluent B), both containing 0.1% of formic acid (v/v). A volume of 5 µl of the prepared samples was used for quantification. Using a Kinetex Biphenyl column (50 × 2.1 mm, 1.7 µm) (Phenomenex, Aschaffenburg, Germany), the flow rate was set to 0.45 ml/min with the following gradient: start conditions 95% of eluent A, decreasing to 80% in 3 min followed by a quick decrease to 2% within 0.5 min. These conditions were held for 1 min and switched to the starting conditions for re-equilibration for 1 min. The mass spectrometer was operated in positive electrospray ionization mode with scheduled multiple reaction monitoring. Three MRM transitions were used for both analytes. For quantification, the peak area of the analytes was further integrated and divided by the peak area of the IS.

Cal samples were fitted with a least-squares fit and weighted by  $1/x$ . The limit of quantification of caffeine was  $1.2 \mu\text{M}$ .

### **Testing of neurobehavioral performance and subjective state**

A comprehensive test battery including the following tasks, validated questionnaires, and physiological measures to assess behavioral, subjective, emotional, cognitive and endocrinological markers of sleep inertia was administered immediately upon awakening (07:00–08:00).

#### **Psychomotor vigilance test (PVT)**

Vigilance was assessed with a 10-min version of the PVT (Dorrian et al., 2005) at 02:30 and 07:05 am, i.e. immediately after awakening. The median reaction time (RT) and the numbers of lapses (trials with  $\text{RT} > 500 \text{ ms}$ ) were analyzed.

#### **Positive and negative affective schedule (PANAS)**

The PANAS (D. Watson et al., 1988) was used to assess mood 15 min after awakening (at 07:15 am).

#### **Caffeine acute questionnaire (CAQ)**

To assess specific caffeine-related effects, the CAQ (Rétey et al., 2007) was applied 30 min after awakening. Participants were asked to rate the following items on a 5-point scale (not at all, a little bit, moderate, much, very much); “Are you feeling any of these caffeine-related effects”: “increased vigilance”, “increased wellbeing”, “increased heart rate”, “increased sociability”, “increased motivation to work”, “increased tension”, “increased concentration”, “increased urge to urinate”, “increased shakiness”, “reduced appetite”, “increased energy”, “increased sweating”, “increased self-confidence”, “reduced headache”, “increased anxiety”, “reduced tiredness”, “stimulation”, “increased nervousness”, “reduced boredom” and “increased stomach trouble”.

#### **Modified sleep inertia questionnaire (SIQ)**

We modified the SIQ (Kanady & Harvey, 2015) to assess volunteers’ subjective experience of the awakening process. The original version of the SIQ represents a trait inventory, in which participants are instructed to rate the quality of their awakening process during the last week, namely on physiological, emotional, cognitive and behavioral levels. Thereby, the inventory instruction reads as follows: “On a typical morning in the past week, after you woke up, to what extent did you, for example, have problems to get out of bed” (possible ratings: 1 = not at all, 2 = a little, 3 = somewhat, 4 = often, 5 = all the time). For the present study, we rephrased the inventory’s instruction to gain state information of the wake-up process of the experimental morning (rather than trait information of the last week), to analyze the acute effects of our pulsatile-release formula. The instruction was rephrased as follows: “How strong did you feel the following aspects after you woke up this morning compared to a normal morning last week: for example, have problems to get out of bed” (possible ratings:  $-3 =$  extremely less,  $-2 =$  much less,  $-1 =$  a

little bit less, 0 = same, 1 = a little bit more, 2 = much more, 3 = extremely more). For our purpose, the modified version of the SIQ was renamed to Acute Sleep Inertia Questionnaire (ASIQ) and was administered at 07:45.

### **N-back task**

At 7:47 the n-back task was executed in 1-, 2- and 3-back versions (Kirchner, 1958). Over a period of 7 min, a random series of letters were displayed and subjects were instructed to press a key when the currently displayed letter corresponded to the previous (1-back), the penultimate (2-back) or the antepenultimate (3-back) letter, respectively. The reaction times and the number of correct and incorrect answers were assessed.

### **d2-task**

Finally, the d2-task, a neuropsychological measure of selective and sustained attention and visual scanning speed (Brickenkamp, 2002), was administered at 07:55. In this task, participants were instructed to cross out any letter “d” with two marks above it or below it in any order. The surrounding distractors were either a “p” with two marks or a “d” with one or three marks. For each line (14 lines in total), subjects were given 20 s to mark all “d” with two marks and then instructed to proceed to the next line. The number of correct and incorrectly crossed characters were determined.

All questionnaires as well as the n-back and d2 tasks were administered as paper–pencil versions.

### **Cortisol awakening response (CAR)**

Cortisone-D<sub>7</sub> was purchased from Sigma Aldrich (Buchs, Switzerland) and <sup>13</sup>C<sub>3</sub> -cortisol was purchased from Isoscience (Ambler, USA). Saliva of each subject was sampled at time points 07:00 (immediately after awakening), 07:15, 07:30, 07:45, and 08:00. Participants were instructed to chew the swab for 60s and then return it into the Salivette ® tube (Sarstedt, Germany). After sampling, tubes were immediately stored on ice until final storage at –80 °C. For cortisol detection, tubes were defrosted and centrifuged for 5 min at 5000 rpm to yield clear saliva in the conical tube. Two subjects had to be excluded, as the amount of saliva yielded from the swabs was insufficient. Then, the swab was removed and the yielded saliva was spiked with 50 µl IS (0.1 ng/µl Cortison-d) for further analysis. A fully automated supported liquid extraction (SLE) was carried out by transferring 265 µl saliva into a column rack (24 × 6 ml) from Biotage ® Extrahera (Biotage, Uppsala, Sweden) and adding 300 µl water to the sample. After mixing the extracts were automatically loaded onto Isolute SLE + columns and allowed to absorb for 5 min. Analytes were then eluted two times with 1.5 ml ethyl acetate with a waiting time of 5 min in-between. The extracts were dried in a Turbovap ® (Biotage, Uppsala, Sweden) at 35 °C. The dry residues were resuspended using 150 µl methanol and 350 µl ammonium formate (5 mM) solution, which was used for liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis following a recently published method using <sup>13</sup>C<sub>3</sub> -labeled cortisol as surrogate analyte for calibration (Binz et al., 2016). The saliva samples were analyzed on an LC–MS/MS system that consisted of a Shimadzu Prominence UFLC (Shimadzu, Kyoto, Japan) high pressure liquid-chromatography (HPLC) system coupled to a Sciex QTRAP ® 6500 + linear ion trap quadrupole mass

spectrometer (Sciex, Darmstadt, Germany). 10 µl of the samples were injected onto a Phenomenex® Kinetex® C 18 column (2.6 µm, 50 × 2.10 mm). The mobile phase consisted of 10 ml ammonium formate (1 M) and 2 ml formic acid in 2 l water (A) and 10 ml ammonium formate (1 M) in 2 l methanol (B). The flow rate was 0.3ml/min and the temperature of the column oven was set to 40°C. The quantification was achieved by using the mass spectrometer in multiple reaction monitoring (MRM) with an ion spray voltage of -4500 V. Cortisol was measured as formic acid adduct [(M-H) + 46] - in negative electrospray ionization mode. The method was validated according to the guidelines of the German Society of Toxicology and Forensic Chemistry (GTFCh). The calibration was prepared by adding 13 C 3 -cortisol to saliva in the concentration range of 0.55 nmol/ml up to 55 nmol/ml. QC samples were prepared in low concentrations (1.5 nmol/l). The limit of detection for cortisol was 0.55 nmol/l and the limit of quantification was 1.1 nmol/l.

### **Polysomnography**

As in previous studies (Dornbierer et al., 2019; Holst et al., 2017; Hubbard et al., 2020; Weigend et al., 2019), sleep in vivo from 03:00 to 07:00 in the pharmacodynamic study was quantified by all-night polysomnography with Rembrandt® Datalab (Version 8; Embla Systems, Planegg, Germany). in vivo Sleep pressure after awakening was also assessed by determining the participants' sleep onset latency (SOL) and sleep patterns during a nap opportunity starting at 08:00 (Fig. 17). The recording setup consisted of 10 EEG electrodes (Fp1, Fp2, F3, F4, C3, C4, P3, P4, O1, O2) according to the 10–20 system (Jasper, 1958), a bipolar electrooculogram (EOG), a submental electromyogram (EMG) and an electrocardiogram (ECG). The individual EEG electrode coordinates were marked by cutting a few hairs at the electrode positions, to ensure that the electrodes were placed at the very same place in both experimental conditions.

All data were recorded with dedicated polysomnographic amplifiers (Artisan®, Micromed, Mogliano Veneto, Italy). As in previous studies of the lab (Dornbierer et al., 2019), the analog signals were conditioned by a high-pass filter (EEG: -3 dB at 0.15 Hz; EMG: 10 Hz; ECG: 1 Hz) and an antialiasing low-pass filter (-3 dB at 67.2 Hz), digitized and stored with a resolution of 256 Hz (sampling frequency of 256 Hz). The data of one participant was excluded from the nocturnal sleep analyses, due to insufficient EEG quality.

### **Visual sleep stage scoring**

Sleep variables were visually scored based on 30-s epochs according to the criteria of the American Academy of Sleep Medicine (Berry et al., 2017). For sleep scoring, the C3-A2 derivation was used. Movement- and arousal-related artifacts were visually identified and excluded from the analyses. The following sleep variables were computed: time spent in (i) wakefulness (Wake), (ii) stage 1 (N1), (iii) stage 2 (N2), (iv) stage 3(N3), (v) stage REM sleep, (vi) sleep onset latency (SOL = time between lights-off and first occurrence of N1), (vii) sleep efficiency (SEFF = [TST/TIB]\*100%; TST = time spent in N1, N2, N3 and REM sleep; TIB = time between lights-off and lights-on), and (viii) pre-awakening stages wake, N1, N2, N3 and REM sleep.

### **Statistical analyses**

Independent linear mixed-effects models, with condition (caffeine vs. placebo) and time point (02:30 and 07:05 for PVT analyses; 07:00, 07:15, 07:30, 07:45 and 08:00 for CAR analyses) as

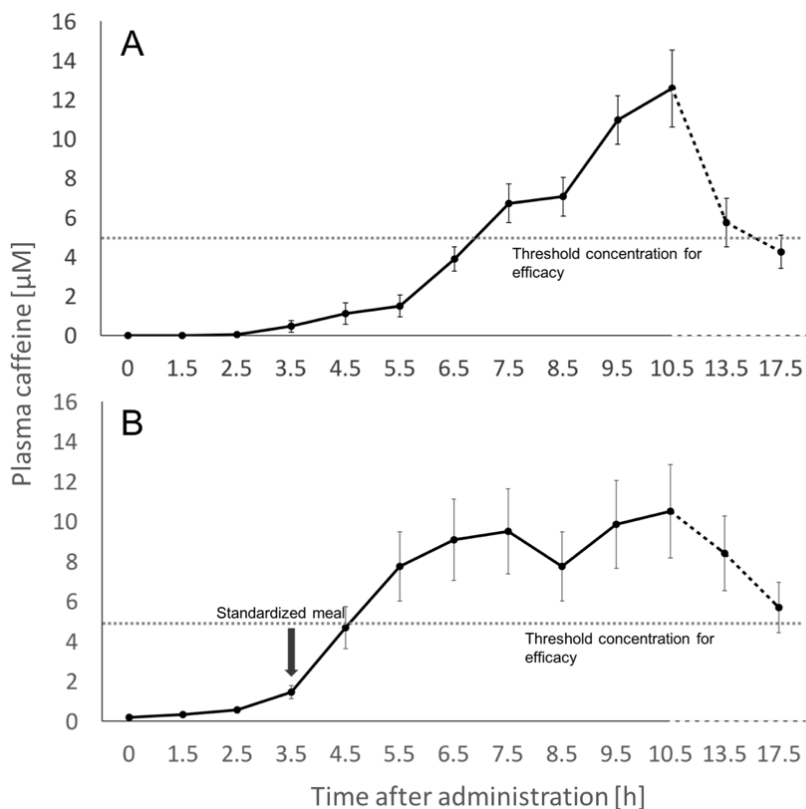


Figure 18: Evolution of the caffeine plasma concentration over time for the in vivo validation study (A) and the pharmacodynamic study (B). Black dots indicate mean caffeine plasma concentrations, error bars indicate standard errors (SEM). The horizontal dashed line at 5  $\mu\text{M}$  indicates the threshold concentration of caffeine efficacy. Time point '0' on the x-axis refers to 22:30 when the caffeine was administered. Sleep periods are indicated as hatched areas.

within-subject factors, and subject ID as random effect were employed on 'R' for the analysis of the (1) PVT; (2) PANAS; (3) CAQ; (4) ASIQ; (5) n-back; (6) d2 task; (7) CAR; (8) nocturnal sleep variables; and (9) morning nap variables

(RStudio Version 1.0.136; RStudio, Inc.; R-package "lme4," Version 1.1–15). For all applied models, normal Q-Q plots were applied, demonstrating normality of the residuals. Moreover, the assumption of homoscedasticity and linearity was verified using a Tukey-Anscombe plot (residuals vs. fitted). Post-hoc testing was carried out using the 'R' package emmeans (Version 1.2.1). The p values of the post-hoc tests were corrected for multiple comparison using Benjamini–Hochberg correction of the false discovery rate (Hochberg & Benjamini, 1990). If not noted otherwise, only significant effects and differences are reported.

#### 4.4 Results

##### Caffeine release profiles

The in vivo validation study during sleep revealed a pulsatile-release profile of the administered formulation;  $c_{\text{max}}$  (maximal plasma concentration) was reached after 10.5 h (Fig. 18A). The caffeine curve followed a sustained-release profile, and efficacious plasma levels ( $> 5 \mu\text{M}$ ) were attained after 7h (Beaumont et al., 2001; Lagarde et al., 2000).

Unexpectedly, in the pharmacodynamic study, the caffeine release profile distinctly differed from that in the in vivo validation study. A sustained-release of caffeine started after 3.5h and efficacious plasma caffeine levels were already attained 5h post-administration (Fig. 18B). The premature burst was most likely triggered by gastric movements due to a standardized meal that was served to the study participants 3.5h post-administration in the pharmacodynamic study. This meal was absent in the in vivo validation study.

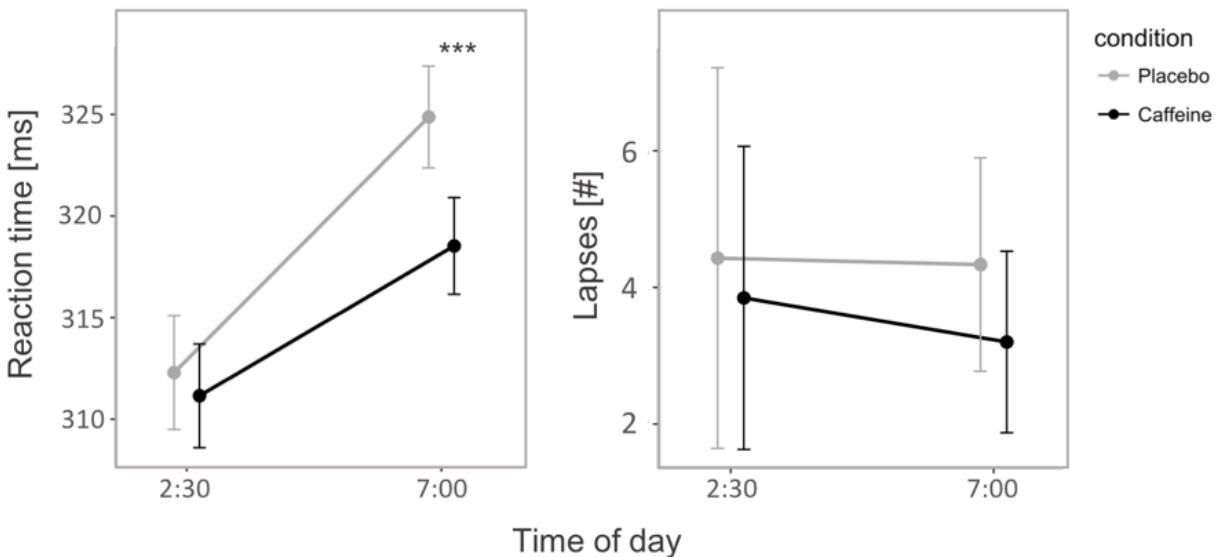


Figure 19: Median PVT reaction time (left) and number of lapses (right) at 2:30 and 7:05. Mean values (dots) and standard error of the mean (vertical lines) are shown. Grey lines indicate the placebo condition; black lines indicate the caffeine condition. \*\*\* $p < 0.001$  (Benjamini–Hochberg corrected).

## Neurobehavioral, subjective, emotional and physiological symptoms of sleep inertia

### Psychomotor vigilance test (PVT)

The statistical analyses of the PVT data revealed a significant condition \* timepoint interaction for median reaction time ( $F = 29.45$ ;  $p < 0.001$ ), such that caffeine improved the PVT median response time by roughly 10 ms when compared to placebo (Fig. 19). On the other hand, the number of lapses remained unaffected ( $F = 1.04$ ;  $p = 0.359$ ).

### Positive-negative affect Scale (PANAS)

Statistical analyses of the PANAS ratings revealed a significant condition \* item interaction ( $F = 8.49$ ;  $p = 0.004$ ;  $\eta^2 = 0.118$ ), such that the engineered caffeine formulation increased positive ratings ( $p < 0.01$ ) and tended to reduce negative ratings ( $p = 0.067$ ) when compared to placebo (Fig. 20).

### Caffeine acute questionnaire (CAQ)

The statistical analyses revealed significantly increased CAQ ratings in the caffeine condition when compared to placebo ( $F = 5.14$ ;  $p = 0.034$ ;  $\eta^2 = 0.196$ ; Fig. 20).

### Acute sleep inertia questionnaire (ASIQ)

The statistical analyses revealed a significant condition effect ( $F = 31.21$ ;  $p < 0.001$ ;  $\eta^2 = 0.175$ ), such that the engineered caffeine-release formula reduced the ratings on all subscales of the ASIQ (behavioral, cognitive, emotional; physiological; Fig. 20). Remarkably, several individuals reported less problems to rise from bed on the experimental mornings compared to a normal

morning in the week preceding the experiment (indicated as negative values in Fig. 20). This notion was particularly true during the caffeine condition (n = number of subjects with negative values: behavioral n = 9, cognitive n = 12, emotional n=9, and physiological n=10) and to a

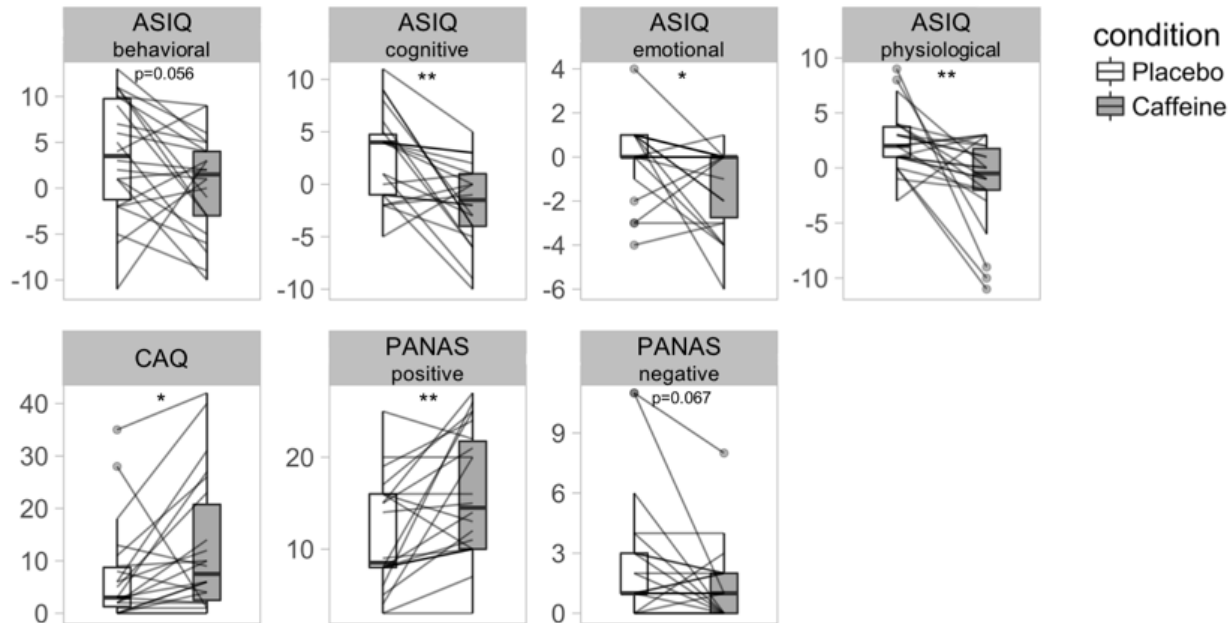


Figure 20: Post-awakening (7:15–8:00) assessments of subjective state. ASIQ = Acute Sleep Inertia Questionnaire (administered at 07:45). CAQ = Caffeine Acute Questionnaire. PANAS-positive = positive affective scale (administered at 07:15). PANAS-negative = negative affective scale (administered at 07:30). \* $p < 0.05$ ; \*\* $p < 0.01$  (Benjamini–Hochberg corrected).

minor degree also during the placebo condition (behavioral n = 5, cognitive n = 7, emotional n = 5, and physiological n = 2).

### N-back and d2 tasks

The statistical analyses of the n-back working memory ( $F = 0.43$ ;  $p > 0.05$ ) and the d2 sustained-attention tasks performance ( $F = 0.29$ ;  $p > 0.05$ ;  $\eta^2 = 0.001$ ) revealed no significant condition effects (data not shown).

### Cortisol awakening response (CAR)

The statistical analyses of the salivary cortisol levels revealed no significant main effect ( $p > 0.05$ ). Nevertheless, post hoc testing revealed significantly increased cortisol levels at 08:00 (60 min post-awakening) in the caffeine condition when compared to placebo condition ( $t = 3.00$ ;  $p < 0.04$ ; Fig. 21).

## **Sleep characteristics**

### **Nocturnal sleep**

Given that the duration of wakefulness was experimentally prolonged prior to the initiation of nocturnal sleep, in both conditions, participants showed a sleep onset latency shorter than 10 min and more than 2 h of deep stage N3 sleep. For all sleep variables analyzed (Table 1), the linear mixed-effects models revealed no significant main effect of “condition” ( $F = 0.56$ ;  $p > 0.05$ ;  $\eta^2 = 0.0025$ ). Nevertheless, post hoc testing with the ‘R’ package emmeans indicated that the time spent in N3 sleep was shorter ( $p = 0.004$ ) in the caffeine condition when compared to the placebo condition (Fig. 22A; Table 1).

### **Pre-awakening sleep**

Statistical analyses of sleep variables during 10 min before scheduled awakening revealed no significant main effect of ‘condition’ ( $F = 0.03$ ;  $p > 0.05$   $\eta^2 = 0.0001$ ) (Fig. 22B; Table 1).

### **Nap sleep opportunity**

The statistical analyses of the sleep variables in the nap opportunity 1 h after scheduled awakening revealed a significant ‘condition’ effect ( $F = 2.09$ ;  $p < 0.01$ ;  $\eta^2 = 0.87$ ), such that sleep onset latency and wakefulness after sleep onset were prolonged. In addition, the time spent in stage N2 was reduced in the caffeine condition when compared to the placebo condition ( $p$  all  $< 0.01$ ; Fig. 22C; Table 1).

## 4.5 Discussion

Here we tested the in vivo drug release profile and (Dornbierer et al., 2021) efficacy to ameliorate morning sleep inertia of a delayed pulsatile-release caffeine formulation administered at bedtime. We found that this innovative approach potentially facilitated the sleep-to-wake transition on neurobehavioral, subjective and physiological markers of sleep inertia in healthy young men following sleep restriction. Most importantly, in contrast to reactive caffeine intake, the novel formula improved vigilance within the first 15 min and mood at 15 min after waking in comparison to placebo.

The in vivo validation study corroborated the intended release profile. As expected based on the in vitro development of the engineered caffeine micropellets, the caffeine curve followed a delayed-release profile, and an efficacious plasma concentration above  $\sim 5 \mu\text{M}$  was attained only after 7 h. Unexpectedly, the release profile of the identical formulation in the pharmacodynamic study was distinctly different from the validation study. Premature caffeine-release started already 3.5 h after drug administration and efficacious plasma levels were attained already 5 h after drug intake.

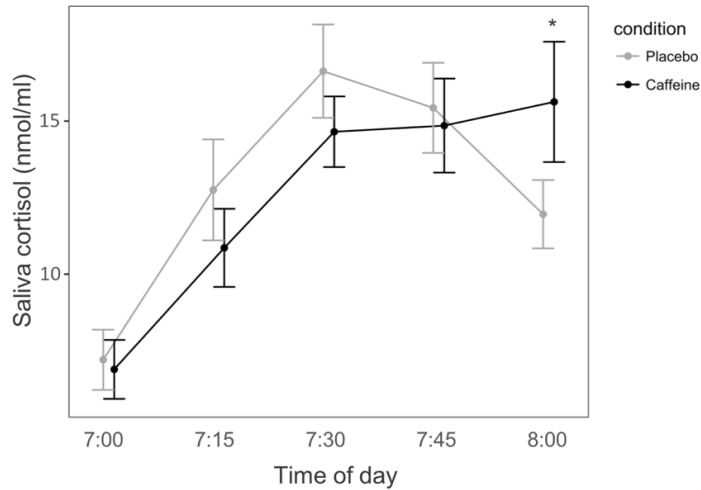


Figure 21: Salivary cortisol awakening response (CAR). Mean salivary cortisol concentration (dots) and standard error of the mean (vertical lines) are shown. Grey lines indicate the placebo condition; black lines indicate the caffeine condition. \* $p < 0.05$  (Benjamini–Hochberg corrected).

Nighttime sleep							
Variables	Placebo		Caffeine		df	t-value	BH.p.value
	Mean	SD	Mean	SD			
Wake [min]	22.333	21.553	38.048	43.399	20	1.511	0.439
SOL [min]	8.810	19.984	11.405	15.449	20	1.084	0.583
N1 [min]	9.714	7.343	10.810	6.501	20	0.641	0.674
N2 [min]	200.333	44.667	206.571	65.912	20	0.590	0.674
N3 [min]	163.190	55.214	128.857	55.156	20	-4.047	0.004
REMS [min]	56.524	34.554	55.857	32.085	20	-0.085	0.933

Nap sleep							
Variables	Placebo		Caffeine		df	t-value	BH.p.value
	Mean	SD	Mean	SD			
Wake [min]	13.082	12.225	25.041	17.018	21	3.727	0.005
SOL [min]	12.873	12.098	23.818	17.220	21	3.165	0.009
N1 [min]	3.032	1.588	2.368	1.910	21	-1.407	0.261
N2 [min]	20.227	9.095	11.414	10.417	21	-3.608	0.005
N3 [min]	3.345	6.320	2.309	4.303	21	-0.925	0.438
REMS [min]	4.923	8.267	3.964	6.318	21	-0.633	0.534

Pre-awakening sleep period							
Variables	Placebo		Caffeine		df	t-value	BH.p.value
	Mean	SD	Mean	SD			
Wake [min]	1.568	2.417	1.955	2.890	21	0.878	0.650
N1 [min]	0.432	0.623	0.409	0.811	21	-0.129	0.898
N2 [min]	4.568	3.364	5.000	3.988	21	0.480	0.795
N3 [min]	1.341	2.427	1.886	2.899	21	0.954	0.650
REMS [min]	2.114	3.214	0.773	1.709	21	-1.693	0.526

Table 2: Visually-scored sleep variables. Means and standard deviations (SD; n = 22) in min in the placebo and caffeine conditions of the nocturnal sleep episode (top), the last 10 min of nocturnal sleep (middle), and the nap sleep opportunity (bottom) are reported. SOL = sleep onset latency; N1, N2, N3, REM = NREM and REM sleep states. df = degrees of freedom. P-values refer to Benjamini–Hochberg corrected post-hoc comparisons.

We suggest that a premature burst of the formula was probably triggered by the meal served to the subjects 3.5 h after caffeine administration (this meal was not served in the validation study). This food intake may have promoted gastric movements that caused faster gastric emptying and provoked the break of the release-modifying polymeric coat due to the physical impact of the peristalsis. Further studies are needed to confirm this hypothesis. In addition, future research may also clarify whether the pharmacokinetics of the engineered caffeine formula systematically differs between sleep and wakefulness.

Despite the premature release of caffeine, the formula ameliorated sleep inertia on the subsequent morning following only 4 h of sleep. The quality of awakening was subjectively improved on behavioral, cognitive, emotional, and physical levels, as indicated on all subscales of the sleep inertia questionnaire. Even though the study participants were sleep restricted, many of them reported less difficulty to rise when compared to a habitual morning, particularly in the caffeine condition.

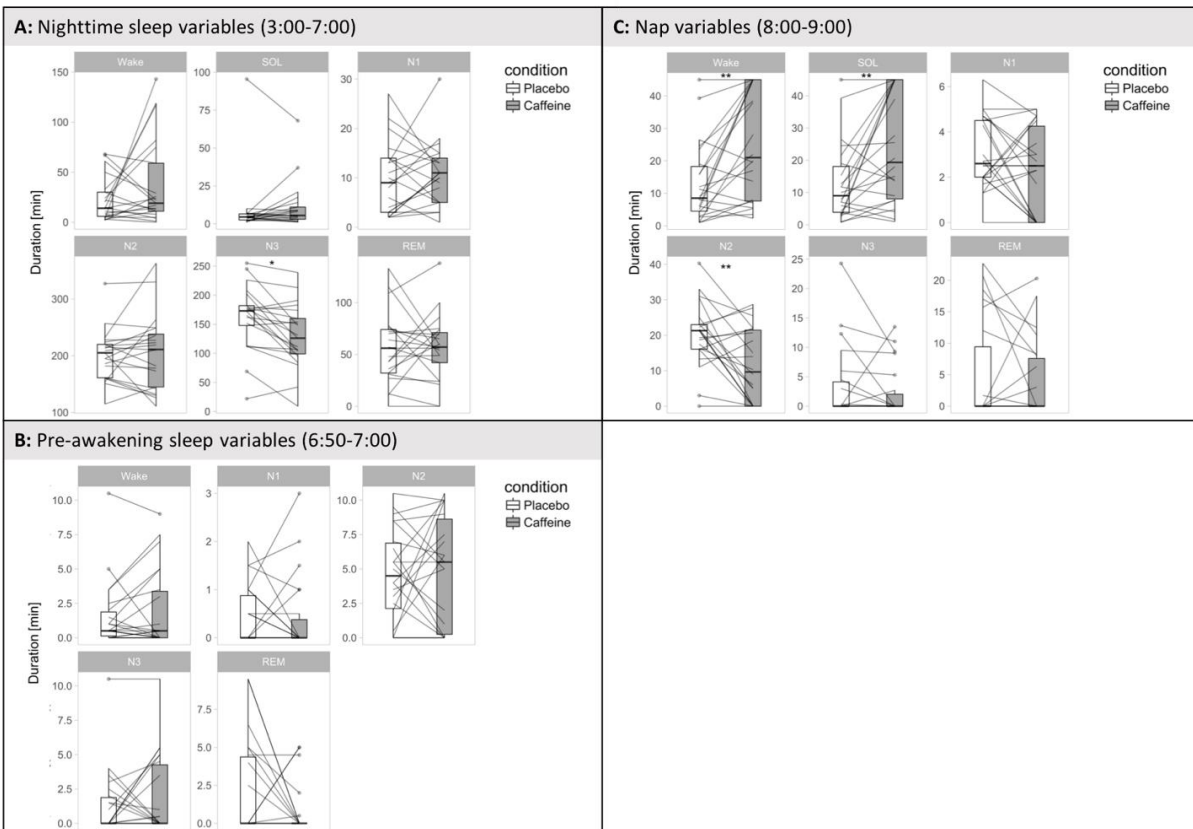


Figure 22: Visually-scored sleep variables in (A) the 4-h nighttime sleep episode (03:00–07:00); (B) the final 10 min before scheduled awakening (06:50–07:00); and (C) the 1-h nap sleep opportunity (08:00–09:00) are shown. SOL, sleep onset latency; N1-3, non-rapid-eye-movement sleep stages N1-3; REM, rapid-eye movement sleep; \*p < 0.05; \*\*p < 0.01 (Benjamini–Hochberg corrected).

The formulation also exhibited mood enhancing properties, as indicated by increased positive and reduced negative ratings on the PANAS. This finding may suggest increased activity of mood-relevant brain structures in the caffeine condition when compared to the placebo condition.

Caffeine blocks adenosine A2A receptors in the nucleus accumbens (Lazarus et al., 2011), a core region of the mesolimbic dopamine network that is essential for the generation of positive mood and reward (Nestler & Carlezon, 2006). We speculate that caffeine-induced dopamine release in the nucleus accumbens (Solinas et al., 2002) could contribute to the post-awakening mood enhancement when compared to placebo. Other possible mechanisms include increased preparatory attention for rewarding stimuli (van den Berg et al., 2021) or direct interaction with sleep-wake regulatory pathways such as the circadian clock (Trautmann et al., 2020). The mechanistic underpinnings of the benefit of the pulsatile caffeine-release formulation on subjective state will have to be clarified in future studies.

The arousal effect of caffeine relies on the competitive antagonism of central nervous system adenosine receptors that contribute to the regulation of sleep intensity and sleep need (Landolt, 2008). Sleep inertia, in particular upon sleep restriction, was previously proposed to reflect 'adenosine left overs' that were insufficiently removed during sleep (Hilditch & McHill, 2019; Radulovacki et al., 1982). Consistent with this view, the primary beneficial effects of the tested caffeine release formula were not restricted to subjective state but included improved vigilance as manifested by faster PVT reaction times when compared to placebo. This finding is important because after nocturnal sleep, no easy applicable proactive or efficacious reactive countermeasure to impaired neurobehavioral performance due to sleep inertia is currently available (Hilditch et al., 2016). Previous work suggested reduced lapsing on the PVT immediately after waking from a 30-min coffee-nap ending at 04:00 (Centofanti et al., 2020) and from repeated 2-h sleep opportunities during prolonged sleep deprivation (Van Dongen et al., 2001). In the present study, we observed no changes in the number of PVT lapses. The differences in the experimental protocols, as well as the caffeine dosages and application forms may underlie the discrepancy. Furthermore, we found no improvement in cognitively more demanding tasks such as sustained selective attention and visual scanning speed (assessed with the d2-task) and working memory and executive functioning (n-back task). These tasks were administered > 45 min after waking. Rested baseline measurements would be necessary to determine whether performance on these tasks at the time of their administration was impaired by sleep inertia and could be improved with the intervention tested.

After caffeine, the cortisol level on the CAR was increased 1 h after wake-up when compared to placebo. This finding supports the hypothesis that caffeine stimulates cortisol secretion and augments the CAR upon waking. The wake-up-related cortisol secretion was previously suggested to reflect a hormonal wake-promoting signal (Boehringer et al., 2015). Nevertheless, neither the peak cortisol concentration nor the area-under-the-curve were affected by caffeine, suggesting that a direct association between reduced sleep inertia within the first 15 min of waking and HPA-axis activity is rather unlikely. Recent studies in rats revealed that overexpression of adenosine A 2A receptors may contribute to glucocorticoid receptor dysfunctions in aged animals and that caffeine may re-sensitize glucocorticoid receptors in the hypothalamus and restore HPA-axis function (Batalha et al., 2016). Although the observed effect on the CAR in our young healthy sample was subtle, it may be speculated that a delayed-release caffeine formulation may promote

cortisol release and glucocorticoid receptor functioning in susceptible individuals (Nader et al., 2009).

Due to the sustained-release profile of the engineered formula, the blood caffeine concentration remained within an efficacious dose range until 17.5 h after administration. Consistent with this pharmacokinetic profile, the increased wake time after sleep onset, the prolonged sleep latency and the reduced N2 sleep duration during the 1-h nap after awakening support the notion that sustained low-dose caffeine administration improved post-awakening vigilance (Van Dongen et al., 2001). The premature high concentration of caffeine during nocturnal sleep most likely also underlies the ~ 17-min reduction in deep N3 sleep observed during the main sleep episode. Such a reduction in deep sleep would hamper the applicability of this novel pulsatile-release caffeine formula. Ongoing research employing quantitative sleep EEG analyses as a function of caffeine levels during sleep, as well as follow-up sleep studies will determine whether sleep is also disturbed without intra-night food intake. With respect to the reduced sleep depth, it seems unlikely that the mitigated sleep inertia after waking depended on the reduced duration of N3 sleep because this sleep state did not differ between the conditions during the final 10 min before scheduled awakening.

Taken together, this proof-of-concept investigation demonstrates that a timed pulsatile-release caffeine system ingested at bedtime can potently attenuate neurobehavioral, subjective, emotional and physiological manifestations of morning sleep inertia in sleep-restricted healthy young men. These findings cannot be generalized because only a single dose, only healthy men, and individuals irrespective of their caffeine sensitivity were studied. Nevertheless, if future research supports these conclusions and further improves the drug-release profile of the engineered formula, time-controlled caffeine administration may be developed as an add-on therapy to mitigate impaired morning state and vigilance in people suffering from excessive sleep inertia, which is highly prevalent in on-call and shift work settings, as well as in patients with neurological, neuropsychiatric, and circadian-rhythm sleep–wake disorders.

## 5 Discussion

### 5.1 Summary

The present dissertation explored the effects of caffeine on neurocognitive performance, sleep physiology, and cardiac autonomic activity in healthy individuals under conditions of sleep restriction. Regular coffee was found to mitigate cognitive decline associated with sleep restriction in participants with genetic predispositions to caffeine sensitivity. Participants who consumed regular coffee performed better on tasks measuring sustained attention, selective attention, and executive control compared to those who consumed decaffeinated coffee. Individuals carrying certain genetic polymorphisms, such as in the ADORA2A gene (Rétey et al., 2007), exhibit heightened sensitivity to caffeine's stimulant effects, enabling them to better counteract the impairment in cognitive performance due to sleep loss. However, the beneficial effects of caffeine on cognitive performance were temporary, as they diminished on the last day

of sleep restriction, indicating the transient nature of caffeine's ability to counteract cognitive impairment due to sleep loss.

In terms of physiological effects, caffeine consumption had a dose-dependent impact on quantitative sleep measures, specifically reducing EEG delta power during non-REM, which suggests shallower or less restorative sleep. Additionally, caffeine increased high-frequency power in heart rate variability, reflecting heightened parasympathetic activity during sleep. In this chapter a clear concentration-dependent relationship between plasma caffeine levels, reductions in EEG delta power, and changes in heart rate variability was established. This suggests that caffeine's impact on sleep quality and autonomic function is closely tied to its dosage and timing of intake.

Chapter 4 examined the impact of a novel pulsatile-release caffeine formulation designed to improve the transition from sleep to wakefulness, thereby reducing sleep inertia. This new formulation improved reaction times on vigilance tasks, enhanced positive affect, extended the cortisol awakening response, and delayed the onset of nap sleep compared to a placebo.

## 5.2 Attention and neurocognitive performance

Building upon the established use of caffeine to counteract the negative effects of sleep restriction on cognitive performance, the findings align with studies showing that during acute sleep deprivation - such as one night of sleep loss - caffeine effectively combats sleepiness and enhances alertness and vigilance (Gottselig et al., 2006). Specifically, improving performance on tasks requiring sustained attention, like the Psychomotor Vigilance Test (Hudson et al., 2020; Urry & Landolt, 2015). In acute scenarios, caffeine enhances response times and accuracy on tasks that measure attentional performance, which are typically degraded by sleep loss (Bianchi, 2014). An improvement is present in the first few days of sleep restriction when cognitive decline is not yet severe, allowing caffeine to effectively sustain cognitive performance (Demiral et al., 2020). However, its impact on higher-order cognitive functions - such as decision-making, memory, and executive functions - is limited after acute sleep deprivation (Snel & Lorist, 2011b; Urry & Landolt, 2015). Chronic caffeine intake also alters neural plasticity and synaptic function, as seen in studies with rats, where it prevented memory impairments and synaptic plasticity loss induced by sleep deprivation. This effect was linked to the modulation of signaling pathways associated with synaptic strength and memory consolidation, such as the phosphorylation of CaMKII in the hippocampus (Alhaider et al., 2011).

## 5.3 Chronic sleep restriction, tolerance, and the declining effects of caffeine

Consuming caffeine shortly before attempting recovery sleep can disrupt sleep quality, leading to reduced total sleep time and alterations in sleep architecture, such as increased light sleep and decreased deep sleep (Belenky et al., 2003). In the context of chronic sleep restriction involving multiple nights of insufficient sleep, it has been shown that caffeine's effectiveness on subjects without a genetic predisposition of caffeine sensitivity diminishes over time (Doty et al., 2017). Initially, caffeine may help maintain alertness and cognitive performance, but these benefits wane over several days due to the accumulation of sleep debt and the development of tolerance to caffeine's effects (Lin et al., 2024). Studies have found that with fixed daily doses of

caffeine, its positive effects on alertness and performance can disappear after the third day of sleep restriction (Doty et al., 2017).

Moreover, chronic caffeine consumption during sleep restriction may negatively affect subsequent recovery sleep, manifesting as prolonged sleep latency, slower return to baseline alertness, and altered sleep architecture, including reduced deep sleep (Clark & Landolt, 2017; Snel & Lorist, 2011b). This suggests a physiological cost associated with chronic caffeine use during sleep restriction, potentially linked to changes in adenosine receptor density and neural plasticity, as well as a delayed return to baseline levels of subjective sleepiness (Boulenger et al., 1983; Elmenhorst et al., 2007).

#### 5.4 Individual differences and genetic predispositions

Significant individual variability exists in both vulnerability to sleep loss and responsiveness to countermeasures like caffeine (Rétey et al., 2007; Van Dongen et al., 2003). This variability results from a complex interplay of factors, including genetics - such as polymorphisms in genes related to adenosine and dopamine signaling - habitual caffeine intake, circadian preference (chronotype), and task-specific demands (Erblang et al., 2019; Urry & Landolt, 2015). While caffeine's antagonism of adenosine receptors temporarily offsets sleepiness, it does not fully restore cognitive performance to baseline levels, particularly during chronic sleep restriction (James & Gregg, 2004). Recovery sleep, important for restoring cognitive function after both acute and chronic sleep deprivation, highlights that caffeine should not be considered a long-term solution for performance decrements induced by sleep loss (McLellan et al., 2016), as attention is particularly vulnerable to such deprivation. This impairment cascades to affect higher-level functions like decision-making, supporting the hierarchical view of cognitive capacities where foundational processes such as attention and memory are necessary for complex tasks (Bianchi, 2014). Neuroimaging studies further indicate that sleep deprivation primarily disrupts lower-level processes, such as visual attention, which then impacts more advanced cognitive functions (Hudson et al., 2020).

It is important to distinguish between different aspects of attention; for instance, while vigilant attention deteriorates with sleep loss, attentional control appears to be a distinct process with potentially different vulnerabilities (Hudson et al., 2020).

Vigilant attention is particularly susceptible to sleep deprivation, a vulnerability prominently illustrated by the PVT, which reliably reveals performance decrements with sleep loss (Dijk & Landolt, 2019). This susceptibility is attributed to factors such as the accumulation of adenosine and affects areas like the basal forebrain responsible for maintaining wakefulness (Borea et al., 2018). Sleep deprivation also induces wake state instability, characterized by fluctuating arousal and brief lapses in attention or "microsleeps," contributing to performance variability on tasks requiring sustained vigilance (Van Dongen et al., 2003). Additionally, time-on-task effects, where maintaining focus over extended periods leads to a decline in vigilant attention known as the vigilance decrement, are amplified by sleep loss (Hudson et al., 2020).

Impairments observed in tasks during sleep deprivation may not always stem directly from impaired attention, highlighting the necessity for careful task decomposition and analysis. While some higher-order functions like working memory are consistently affected by sleep loss, there is potential resilience in specific sub-components, such as those related to motor action

planning or resisting proactive interference (Fournier et al., 2020). Similarly, the effectiveness of caffeine varies across different cognitive tasks. Caffeine consistently improves vigilance but has a less consistent impact on complex functions like decision-making or cognitive flexibility, depending on the specific task and underlying neural systems involved (Bianchi, 2014; Urry & Landolt, 2015).

### 5.5 Caffeine's functions on the autonomic nervous system

Caffeine's effects on heart rate and blood pressure are complex and can vary depending on several factors, including dosage, individual physiology, and habitual intake. Although caffeine is typically linked to an increase in heart rate and blood pressure due to its stimulatory effects, chapter 3 elaborated on the observed decrease in heart rate following caffeine consumption. One primary mechanism by which caffeine can increase blood pressure is through adenosine receptor antagonism. Adenosine can act as a vasodilator via the adenosine 2A receptor, promoting blood vessel relaxation (Riksen et al., 2011). By blocking adenosine's vasodilatory effects, caffeine leads to vasoconstriction, increasing total peripheral resistance and elevating blood pressure.

Caffeine, depending on dosage and sensitivity of the subjects, can trigger the release of catecholamines, such as epinephrine from the adrenal glands, which causes vasoconstriction and consequently raises blood pressure by increasing peripheral vascular resistance. Studies have shown that caffeine leads to acute increases in systolic and diastolic blood pressure, primarily due to heightened vascular resistance rather than enhanced cardiac output (Pincomb et al., 1985). As blood pressure rises, the baroreflex detects this change through receptors in areas like the carotid sinuses and aortic arch. This reflex activates the parasympathetic nervous system to counteract the elevated blood pressure, resulting in a decrease in heart rate, known as reflex bradycardia. The baroreflex response thus can lead to a reduction in heart rate, even as blood pressure increases (Mosqueda-Garcia et al., 1990). Furthermore, this baroreceptor-mediated reflex explains the observed decrease in heart rate following caffeine intake. Studies in children showed similar effect, where elevated blood pressure prompted a compensatory heart rate reduction via the baroreceptor mechanism (Turley et al., 2017).

### 5.6 Caffeine formulations, pharmacokinetics, and study variability

There is evidence that baroreflex activation, enhanced by parasympathetic nervous system activity, may be reflected in increased heart rate variability, particularly in the high-frequency band, which is a marker of parasympathetic influence on the heart. This observation supports the idea that caffeine might, promote parasympathetic activity, leading to a lower heart rate (Crooks et al., 2019).

Long-term, habitual caffeine consumption can lead to complex adaptations in the body's adenosine system. Chronic caffeine exposure may result in an upregulation of adenosine receptors, increasing their density and making the body more sensitive to adenosine's effects (Reichert et al., 2022). This adaptation could counteract caffeine's stimulatory actions, potentially leading to a lower resting heart rate in habitual caffeine consumers due to the enhanced effects of adenosine when caffeine is not present.

Individual variability plays a significant role in caffeine's effects on heart rate and blood pressure. Higher doses are more likely to trigger increases in both heart rate and blood pressure due to more potent sympathetic stimulation and potential direct effects on the heart and blood vessels. Lower doses might have milder or even opposite effects, potentially leading to a decrease in heart rate through enhanced parasympathetic activity or primarily influencing blood pressure through adenosine receptor antagonism in the vasculature (de Paula Lima & Farah, 2019). Habitual caffeine intake affects sensitivity to caffeine's acute effects. Regular consumers often develop tolerance, diminishing caffeine's impact on heart rate and blood pressure. A person accustomed to high caffeine intake might experience minimal or no changes in these parameters after consuming their usual dose. In contrast, someone who rarely consumes caffeine might exhibit more pronounced increases in both heart rate and blood pressure upon intake (Riksen et al., 2011).

Genetic variations also significantly contribute to individual responses to caffeine (Clark & Landolt, 2017). These genetic differences can affect caffeine metabolism and sensitivity to its effects on adenosine receptors (Lazarus, Chen, et al., 2019). Some individuals with CYP1A2 variants, which make them "fast metabolizers" of caffeine, experience faster clearance and potentially less pronounced cardiovascular effects (Guessous et al., 2012). Conversely, "slow metabolizers" might experience more prolonged and intense effects from the same caffeine dose, including greater increases in heart rate and blood pressure (Soares et al., 2018). The body's natural circadian rhythm, which influences hormone levels and physiological processes throughout the day, can interact with caffeine's effects. Caffeine consumed in the evening, when the body has a high propensity for sleep and adenosine levels are high, might have different effects on heart rate and blood pressure compared to caffeine ingested in the morning (Weibel et al., 2020). The time of day can modulate the body's responsiveness to caffeine, potentially amplifying or diminishing its cardiovascular effects. Sleep deprivation can significantly modulate the body's response to caffeine, affecting its cardiovascular effects. In a sleep-deprived subject, with accumulated levels of adenosine, an altered balance between sympathetic and parasympathetic nervous system activity is present (Porkka-Heiskanen & Kalinchuk, 2011). In this state, caffeine's blockade of adenosine receptors may be less effective in stimulating the sympathetic nervous system, leaving a heightened parasympathetic activity and a decrease in heart rate (Crooks et al., 2019).

Caffeine affects physiological responses differently based on its formulation and individual factors. Various administration routes - including capsules, tablets, energy shots, pouches, and chewing gums - exhibit similar overall bioavailability but differ in absorption rates, with liquid forms showing superior absorption within the first 15 minutes (Grzegorzewski et al., 2022). Different formulations can alter the rate at which caffeine enters the bloodstream and reaches its target sites, influencing the onset, intensity, and duration of its effects. Immediate-release formulations, such as those found in a standard cup of coffee or caffeine tablets, lead to relatively rapid absorption of caffeine, resulting in a quicker onset of effects but a shorter duration of action. In contrast, sustained-release formulations, present in some caffeine capsules, are designed to release caffeine gradually over an extended period. This leads to a slower rise in blood caffeine levels, potentially resulting in a more sustained effect and longer duration of action but with a delayed onset compared to immediate-release formulations (Bianchi, 2014).

Comparing caffeine studies can be challenging due to variations in caffeine dose, method and timing of administration, and whether the delivery is acute or sustained release (Smith, 2002). This variability emphasizes how formulation choices can contribute to differing study results regarding caffeine's effects. The rate of caffeine absorption could potentially influence its cardiovascular effects. A rapid influx of caffeine into the system may be more likely to produce a transient spike in blood pressure or heart rate, whereas a more gradual absorption might have milder effects on these parameters (Robertson et al., 1981). Individual responses to caffeine can vary significantly based on genetics, metabolism, tolerance, and other factors. Genetic variations, particularly in the CYP1A2 and ADORA2A genes, influence caffeine metabolism and effects (Fulton et al., 2018; Nehlig, 2018). Age, sex, and diet also contribute to the variability in caffeine's impact among individuals (Magkos & Kavouras, 2005; Urry & Landolt, 2015).

## 5.7 Mechanisms of adenosine clearance during sleep

As discussed in Chapter 2, the findings show that caffeine intake negatively affects slow-wave sleep by reducing delta activity during non-REM sleep, raising questions about how the restorative nature of this sleep stage is maintained, given that neuronal activity is still present and the brain remains functionally active, which would seemingly continue accumulating adenosine.

However, during non-REM slow-wave sleep, there is a significant reduction in overall brain metabolism and neuronal firing rates, coupled with slow, synchronized neural oscillations (Nir et al., 2013). This decrease in neuronal activity leads to reduced adenosine production, which is typically associated with energy consumption (Porkka-Heiskanen et al., 2002). The lowered metabolic demand during slow-wave sleep allows for the clearance of accumulated adenosine, despite the brain's ongoing activity.

Adenosine levels decrease during sleep through several mechanisms. One is metabolic clearance, where adenosine is taken up by cells via nucleoside transporters and metabolized by enzymes such as adenosine deaminase and adenosine kinase (Reichert et al., 2016).

Additionally, the glymphatic system becomes more active during sleep, enhancing the removal of metabolic waste products, including adenosine, from the interstitial fluid (Xie et al., 2013).

This system leverages the convective movement of cerebrospinal fluid to clear waste, which is more efficient during the reduced neuronal activity of slow-wave sleep.

Glial cells, particularly astrocytes, significantly contribute to sleep regulation and adenosine metabolism. Astrocytes release adenosine during wakefulness, contributing to the build-up of sleep pressure (Halassa et al., 2009). Astrocytes and neuronal circuits also interact on spatial and temporal scales distinct from neuronal activity, allowing for extended control over synaptic and network activity (Schmitt & Wimmer, 2015). During sleep, astrocytes modulate neuronal activity and may aid in the clearance of adenosine, thus participating in the dissipation of sleep pressure. Inhibiting gliotransmission has been shown to attenuate the accumulation of sleep pressure and prevent cognitive deficits associated with sleep loss (Halassa et al., 2009).

Furthermore, sleep deprivation and increased sleep drive reduce interactions between astrocytes and neurons, indicating dynamic alterations in neural-glia interactions during changes in sleep pressure (Vanderheyden et al., 2019).

Rapid eye movement sleep contrasts with non-REM sleep by exhibiting a resurgence of brain activity that closely resembles wakefulness, including desynchronized neuronal firing patterns (Nir et al., 2011). Adenosine clearance may be less efficient during REM sleep due to higher metabolic activity, aligning with observations that REM sleep is less directly involved in the dissipation of sleep pressure (Landolt, 2008).

While generally adenosine promotes sleepiness through action on adenosine A1 and A2A receptors, its effects can vary depending on the brain region and receptor subtype involved (Huang et al., 2014). The expression of adenosine receptors is dynamic and can be modulated by sleep-wake patterns and adenosine levels. For example, chronic caffeine use leads to upregulation of adenosine receptors as a compensatory mechanism in response to prolonged receptor blockade (Elmenhorst et al., 2007). This plasticity suggests that receptor expression adjusts to maintain homeostasis in response to changes in adenosine availability.

## 5.8 The transition from sleep to wakefulness

The downstream effects of adenosine receptor activation or expression are also significant after waking, particularly in relation to sleep inertia, which reduces alertness and impairs cognitive functions. In Chapter 4, a new caffeine formulation was discussed that would combat the symptoms of sleep inertia. This suggests that adenosine's role in cognitive performance extends beyond sleep promotion, influencing the difficulties encountered during the transition from sleep to wakefulness. Sleep restriction often leads to an increase in the proportion of slow-wave sleep (Landolt et al., 2004). This compensatory increase is the response to elevated adenosine levels and recover from sleep debt by prioritizing the most restorative stages of sleep. Abrupt awakening from deep sleep tends to enhance symptoms of sleep inertia. This is supported by studies that found that sleep inertia is more severe when slow-wave sleep, compared to REM sleep (Van Dongen et al., 2001). The transition from the high-amplitude, low-frequency brain activity of slow-wave sleep to wakefulness is more challenging, leading to greater cognitive impairment and subjective feelings of grogginess upon awakening.

By blocking adenosine receptors, caffeine reduces the inhibitory effects of adenosine on neuronal activity, promoting wakefulness and enhancing cognitive performance upon awakening (Landolt et al., 2004). Sleep inertia is also influenced by other factors, including prior sleep duration and quality, circadian rhythms, and individual susceptibility (Tassi & Muzet, 2000). Sleep restriction exacerbates sleep inertia by increasing sleep pressure and altering sleep architecture, resulting in deeper sleep stages from which awakening is more difficult (Doran et al., 2001). The interplay between elevated adenosine levels, increased slow-wave sleep, and the challenging transition from slow-wave sleep to wakefulness underlies the potentiation of sleep inertia following sleep restriction.

By antagonizing adenosine's action on its receptors, caffeine suppresses this signal, leading to a subjective feeling of alertness despite the objective build-up of sleep pressure (Landolt, 2008). In this context, caffeine's ability to disrupt sleep patterns, extend sleep latency, and make it more difficult to fall asleep again is viewed as a beneficial effect.

Although, chronic daily consumption of caffeine can lead to adaptations in the sleep-wake system, potentially making its disruptive effects on sleep less pronounced (Reichert et al., 2022). Some studies have found that habitual caffeine intake does not significantly reduce slow-

wave activity in non-REM sleep, although it may lower sigma activity, another EEG marker associated with sleep spindles (Weibel et al., 2020). This suggests that chronic caffeine use might lead to a shift in sleep architecture, favoring lighter sleep stages even if overall slow-wave activity remains relatively unaffected.

Chronic caffeine use can also result in tolerance, where higher doses are needed to achieve the same stimulating effect (Weibel et al., 2020). Abrupt cessation after prolonged intake may lead to withdrawal symptoms such as increased sleepiness, impaired vigilance, and a quicker onset of slow-wave sleep - indicative of a rebound increase in sleep pressure. These withdrawal effects highlight the adaptation to the constant presence of caffeine and its influence on sleep-wake mechanisms. While caffeine can temporarily alleviate sleepiness and performance deficits caused by sleep restriction, it does not address the underlying physiological need for sleep (Urry & Landolt, 2015).

## 5.9 Additional factors influencing sleep and wakefulness and concluding remarks

Although the accumulation of adenosine throughout the day is believed to promote sleep by inhibiting neuronal activity, particularly in areas such as the basal forebrain that regulate cortical vigilance (Porkka-Heiskanen et al., 2002), it is not the sole factor. Other mechanisms and processes also play a role in the regulation of sleep and wakefulness. Melatonin levels, which increase in the evening driven by the circadian rhythm, also play a significant role in promoting sleep. Melatonin works alongside adenosine, and the interaction between genetic factors and melatonin levels can influence sleep quality and the timing of sleep onset (Tartar et al., 2021). Additionally, the type of neuronal activity during wakefulness affects the buildup of sleep pressure. Specific patterns of neuronal activity, particularly those associated with active wakefulness, contribute to the elevation of adenosine and thereby increase sleep pressure (Wigren et al., 2007).

Overall, this work underscores the complex interplay between caffeine, sleep physiology, cardiac autonomic activity, and cognitive performance. The findings emphasize that while caffeine can temporarily alleviate some negative effects of sleep restriction, its benefits are transient and heavily influenced by individual genetic factors. The dose-dependent impacts on sleep architecture and heart rate variability highlight the necessity of careful consideration regarding caffeine dosage and timing. The positive results of the novel pulsatile-release caffeine formulation in reducing sleep inertia show the potential for optimizing caffeine use in mitigating sleep-related cognitive impairments. These insights contribute to a better understanding of caffeine's complex role but also pave the way for personalized approaches in managing sleep restriction and mitigating its negative effects on cognitive performance, although more research is still needed.

## 6 References

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## 7 Curriculum vitae



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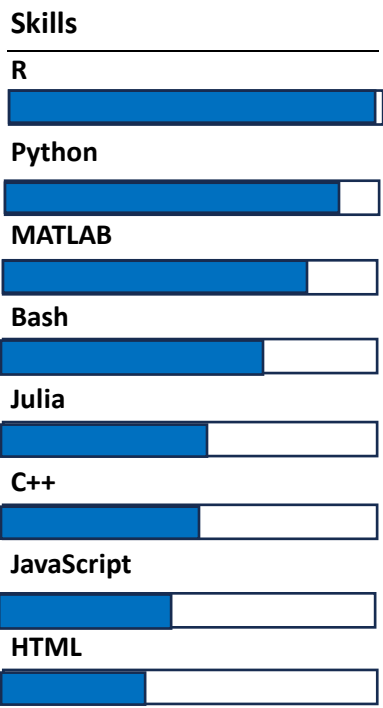
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### About me

Raised and educated in Ticino, the southern region of Switzerland, I am currently finalizing my Ph.D. in sleep research. My interests span over neuroscience, programming, machine learning, aviation, and sports. The onset of the COVID-19 pandemic piqued my interest in server administration allowing me to independently manage and host essential services that support my work, such as platforms for online meetings, data analysis and storage, as well as tools for the visualization of research outcomes.

## Publications

- 2019 Neurophysiological signature of GHB augmented sleep in male healthy volunteers may reflect biomimetic sleep enhancement: a randomized controlled trial. *Neuropsychopharmacology*  
Dornbierer DA, **Baur DM**, Stucky B, Quednow BB, Krämer Th, Seifritz E, Bosch OG, Landolt HP.
- 2019 Nocturnal gamma-hydroxybutyrate reduces cortisol awakening response and morning kynurenine pathway metabolites in healthy volunteers. *Int. J. Neuropsychopharmacology*  
Dornbierer DA, Boxler M, Voegel CD, Stucky B, Steuer A, Binz TM, Baumgartner MR, **Baur DM**, Quednow BB, Kraemer T, Seifritz E, Landolt HP, Bosch OG.
- 2020 Coffee effectively attenuates impaired attention in ADORA2A C/C-allele carriers during chronic sleep restriction. *Progress in Neuro-Psychopharm. and Biol. Psychiatry*  
**Baur DM**, Lange D, Elmenhorst EM, Elmenhorst D, Bauer A, Aeschbach D, and Landolt HP.
- 2021 Cerebral A1 adenosine receptor availability in female and male participants and its relationship to sleep. *NeuroImage*  
Pierling AL, Elmenhorst EM, Lange D, Hennecke E, **Baur DM**, Beer S, Kroll T, Neumaier B, Aeschbach D, Bauer A, Landolt HP, Elmenhorst D.
- 2021 A novel bedtime pulsatile-release caffeine formula ameliorates sleep inertia symptoms immediately upon awakening. *Scientific Reports*  
Dornbierer DA, Yerlikaya F, Wespi R, Boxler MI, Voegel CD, Schnider L, Arslan A, **Baur DM**, Baumgartner MR, Binz TM, Kraemer T, Landolt HP
- 2023 Total Sleep Deprivation Increases Brain Age Prediction Reversibly in Multisite Samples of Young Healthy Adults. *Journal of Neuroscience*  
Chu C, Holst SC, Elmenhorst EM, Foerges AL, Li C, Lange D, Hennecke E, **Baur DM**, Beer S, Hoffstaedter F, Knudsen GM, Aeschbach D, Bauer A, Landolt HP, Elmenhorst D.
- 2023 Nocturnal sodium oxybate increases the anterior cingulate cortex magnetic resonance glutamate signal upon awakening. *Journal of Sleep Research*  
Dornbierer DA, Zölch N, **Baur DM**, Hock A, Stucky B, Quednow BB, Kraemer T, Seifritz E, Bosch OG, Landolt HP.
- 2023 Repeated Caffeine Intake Suppresses Cerebral Grey Matter Responses to Chronic Sleep Restriction in an A1 Adenosine Receptor-Dependent Manner. *Scientific Reports*  
Lin YS, Lange D, **Baur DM**, Foerges A, Chu C, Li C, Elmenhorst EM, Neumaier B, Bauer A, Aeschbach D, Landolt HP, Elmenhorst D.



2023 A genetic variation in the adenosine A2A receptor gene contributes to variability in oscillatory alpha power in wake and sleep EEG and A1 adenosine receptor availability in the human brain. *NeuroImage*  
*Tichelman N, Foerges A, Elmenhorst EM, Lange D, Hennecke E, Baur DM, Beer S, Kroll T, Neumaier B, Bauer A, Landolt HP, Aeschbach D, Elmenhorst D.*

2024 Submitted Concentration-effect relationship of plasma caffeine on EEG delta power and cardiac autonomic activity during human NREM sleep. *Journal of sleep Research*  
*Baur DM, Dornbierer DA, Landolt HP.*

2024 ADHD-related alterations in resting-state EEG-fMRI signal coupling. *NeuroImage Clinical*  
*Michels L, Baur DM, Bollmann S, Ghisleni C, Klaver P, O’Gorman R, Brandeis D.*

**Education:**

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2007-2009	Basic studies in Physics	-	Swiss Institute of technology – ETHZ
2009-2012	Bachelor of Science in Biology	-	Swiss Institute of technology – ETHZ
2012-2015	Master of Science in Biology	-	Swiss Institute of technology – ETHZ
	<i>1<sup>st</sup> Master Project</i>	-	<i>Laboratory of Molecular Neuro-oncology – USZ</i> Angiopietin ligand and receptor expression in mouse gliomas in-vitro and ex-vivo.
	<i>2<sup>nd</sup> Master Project</i>	-	<i>Institute of Molecular Systems Biology – ETHZ</i> Cleavable cross linker for cell surface protein interaction analysis in neurons.
	<i>Master Thesis</i>	-	<i>University Children’s Hospital Zürich – MR research Center.</i> Resting state EEG-fMRI BOLD functional coupling in ADHD.
2016-2018	Research assistant	-	Sleep research center at University Zürich – UZH
2018-today	Ph.D. candidate	-	Sleep research centers at the German aerospace center in Cologne and University Zürich

**Qualifications:**

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2009	Diploma, Fitness Betreuer: Swiss Academy of Fitness and Sports.
2013	Diploma, Berater für Mikronährstoffmedizin.
2015	Diploma, Berater für Anti-Aging und Präventiv Medizin.
2015	Diploma, Fitness Trainer: Swiss Academy of Fitness and Sport.
2015-2019	Traning, Teaching diploma for secondary education – ETHZ
2017-2019	Traning, Applied statistics – ETHZ
2018	Certification, Group Fitness Basics
2018-2020	Diploma, PTA Sport taping expert
2022	Certification, Outdoor group fitness trainer
2023	Certification, Machine learning: Coursera

## Skills:

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Informatic	<i>Programming:</i> R, Python, Matlab, Bash, Julia, C++, HTML, PHP, MySQL, QBasic. <i>Linux server administration:</i> Deploy services via Docker, R shiny, Python streamlit or Flask.
Lab. Tech.	<i>Cell biology:</i> Cell lines/primary cell culture, growth curve, RNA/DNA/Protein extraction, Western blot, qRT-PCR, siRNA, Plasmid DNA transfection, immunocytochemistry and cellular image analysis, luciferase assay, Bradford assay. <i>Mass spectrometry:</i> Quadrupole time of flight (QTOF) liquid chromatography tandem mass spectrometry LC-MS/MS measurements, use of chemicals for sensitivity enhancement and result analysis.
Imaging	<i>Microscopy:</i> Light microscopy, fluorescent/laser confocal microscopy <i>EEG / MRI:</i> functional MRI, EEG, ECG, PSG data acquisition, processing and analysis.
Licences	<i>Drivers licence</i> cat. B, BE, C1, C1E, D1, D1E and boat licence cat. A Private pilot licence and ongoing airline transport pilot licence.
Languages	Languages Native languages: German and Italian; English: Fluent; French: Very good