Journal of Integrative Neuroscience

*J. Integr. Neurosci.* **2026**; 25(8): 46140
https://doi.org/10.31083/JIN46140

*Original Research*

# Age-Dependent Effects of Caffeine on Physiological, Behavioral, and Circadian Gene Changes in Young and Mature C57BL/6 Male Mice

Sookyoung Park[1,†], Zeeshan Ahmad Khan[1,†], AbuZar Ansari[2], Urala Kalasi Apsara Shamali[3,4], Anik Kumar Kar[3,4], Yejin Lee[1,5], Gwangbae Lee[1,5], Jaeyeong Kim[1,5], Semin Son[1,5,6], Yonggeun Hong[1,3,4,7,*]

[1]Department of Physical Therapy, College of Biomedical Sciences & Health, Inje University, 50834 Gimhae, Republic of Korea
[2]Department of Rehabilitation Medicine of Korean Medicine, Dongguk University, 10326 Goyang, Republic of Korea
[3]Department of Digital Anti-aging Healthcare, Graduate School, Inje University, 50834 Gimhae, Republic of Korea
[4]DREAM Core Research Center (CRC), Inje University, 50834 Gimhae, Republic of Korea
[5]Changwon Science High School, Anatomy and Practice, 51371 Changwon, Republic of Korea
[6]Masan Girls High School, 51267 Changwon, Republic of Korea
[7]Department of Rehabilitation Science, Graduate School, Inje University, 50834 Gimhae, Republic of Korea
*Correspondence: yonghong@inje.ac.kr (Yonggeun Hong)
[†]These authors contributed equally.
Academic Editor: Bettina Platt
Submitted: 29 August 2025 Revised: 29 April 2026 Accepted: 11 May 2026 Published: 3 August 2026

**Abstract**

**Background:** Caffeine, a widely consumed psychoactive substance, has known effects on physiological and behavioral processes. Recent studies report that caffeine can influence the circadian rhythm, which is linked to many aspects of physiology, energy metabolism, and homeostasis. Therefore, we examined the arousal effects of caffeine via behavioral tests and assessed its impact on the circadian rhythm by measuring clock gene and clock-controlled gene expression in the brains of mice and Neuro 2a (N2a) cells. **Methods:** Male C57BL/6 mice from two age groups, young (1 month 2 weeks) and mature (10 months 2 weeks), were randomly divided into four subgroups: control, vehicle, caffeine, and caffeine with adenosine (n = 8 each). After inducing fatigue through forced treadmill exercise, we performed balance beam and foot fault tests, and measured the mRNA expression and protein levels of circadian genes (circadian locomotor output cycles kaput (*Clock*), brain and muscle ARNT-like 1 (*Bmal1*), period circadian regulator 2 (*Per2*), cryptochrome circadian regulator 1 (*Cry1*), and *Cry2*) in the brain and N2a cells treated with caffeine. **Results:** Caffeine treatment showed a trend toward reduced body weight gain in both young and mature mice. Behavioral tests indicated improvements in balance and coordination across both age groups. After 16 days of treatment, circadian genes showed significant differences in expression, and adenosine receptor levels changed notably, especially after long-term exposure. In N2a cells, caffeine decreased viability in a dose-dependent manner and affected circadian gene expression. **Conclusions:** The study demonstrates that caffeine influences body weight, behavior, circadian rhythms, and adenosine receptor protein levels, with age-related variations. These findings deepen the understanding of caffeine's effects on physiological and molecular functions and suggest possible age-specific therapeutic implications or precautions related to caffeine intake.

**Keywords:** caffeine; adenosine; dexamethasone; circadian rhythm; homeostasis

## 1. Introduction

Caffeine, one of the most widely consumed psychoactive substances worldwide can acutely stimulate the central nervous system (CNS) and enhance alertness in neuropsychological function [1,2]. Interestingly, chronic caffeine consumption re-wires and optimizes brain readiness for adaptive responses [3,4] rather than stimulating the brain [5]. Approximately 90% of adults in Western societies consume caffeine daily, primarily through coffee, tea, and energy drinks [6,7]. However, its impact on sleep quality and circadian rhythms has become a growing concern.

Research shows that caffeine consumption can delay sleep onset and decrease total sleep time. However, there is also evidence indicating that coffee intake might enhance sleep quality in certain populations. Jaussent et al. [8] found that caffeine intake had a protective effect against insomnia symptoms in mature adults. Similarly, Curless et al. [9] reported that caffeine consumption was not a significant factor in subjective insomnia among elderly people. Furthermore, van der Linden et al. [10] observed that mature females who consumed caffeine experienced fewer sleep disturbances and had better sleep duration compared to those who abstained.

Chronic caffeine consumption can affect neurophysiology, yet it counteracts central fatigue. Alves et al. [11] found that caffeine helps reduce central fatigue by blocking adenosine receptors. Similarly, Davis et al. [12] demonstrated that caffeine delays fatigue during exercise by affecting CNS mechanisms. These studies suggest that caffeine plays a significant and beneficial role in mitigating

central fatigue, particularly in contexts that demand sustained mental and physical performance [13]. Additionally, high doses of caffeine have significant effects on circadian rhythms, as disruptions in circadian rhythms can lead to various neuropsychological impairments [1,14]. Some discussions have linked neuropsychological impairments to learning and behavior issues, suggesting that caffeine intake can be harmful to brain function. However, such disruptions are only seen with toxic doses of caffeine, not with moderate doses taken regularly. Additionally, neurophysiological impairments are also connected to declines in mood and memory. In contrast, there is evidence pointing to a trend where caffeine intake may help preserve memory and mood. For example, Espinosa et al. [15] showed in animal models that caffeine helps maintain memory. Similarly, Zhu et al. [16] highlighted that caffeine intake maintains memory in humans. Additionally, the regular intake of moderate doses of caffeine is associated with the preservation of mood in both animal models [17] and humans [18]. This evidence provides a more balanced discussion on caffeine's impact on brain function, demonstrating its potential neuroprotective benefits when consumed in moderate amounts. The growing body of evidence underscores the need for a deeper understanding of how caffeine affects circadian regulation, neuropsychological changes, and overall physiological function, particularly in light of its widespread consumption.

Circadian rhythms, controlled by an internal biological clock, synchronize many bodily functions with the 24 hr day-night cycle, influencing sleep patterns and physiological and hormonal homeostasis [19]. At the molecular level, circadian rhythms are regulated by a network of clock genes, including transcription factors Circadian Locomotor Output Cycles Kaput (*Clock*) and Brain and Muscle ARNT-Like 1 (*Bmal1*), which mediate transcription of the Period (Period Circadian Regulator [*Per*]*1*, *Per2*, *Per3*) and Cryptochrome (Cryptochrome Circadian Regulator [*Cry*]*1*, *Cry2*) genes. These genes form transcriptional feedback loops to synchronize physiological processes [20]. Studies have shown that caffeine can alter circadian gene expression, with a single dose potentially shifting the timing of core clock genes by 1–2 hr. Caffeine impacts circadian rhythms primarily through its antagonistic effects on adenosine receptors, specifically A1 and A2A receptors [21]. Adenosine, a key modulator of sleep and circadian rhythms, is normally involved in feedback mechanisms that regulate clock gene expression [22]. By blocking adenosine receptors, caffeine increases levels of cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA) and subsequently influences cAMP response element-binding protein (CREB) [23]. This cascade alters the expression of circadian clock genes such as *Clock*, *Bmal1*, *Per2*, and *Cry2*, impacting the synchronization of physiological processes [24]. By disrupting these clock proteins, caffeine leads to altered rhythmic expression patterns [14]. Additionally, caffeine-induced changes in clock gene expression can potentially increase the risk of metabolic disorders [25]. Therefore, caffeine's disruption of adenosine signaling, cAMP/PKA pathways, and clock protein stability emphasizes its significant influence on circadian rhythms and points to the importance of strategies to regulate caffeine intake and its impact on overall health.

Understanding how caffeine interferes with circadian rhythms is vital for grasping its broader health impacts. Caffeine primarily blocks adenosine receptors, which are crucial for sleep regulation and circadian clock function [26]. This blockade disrupts the normal signaling pathways for sleep-wake cycles and circadian gene expression, leading to immediate increases in alertness and longer-term disturbances in physiological processes aligned with circadian rhythms [27]. The effects of caffeine can vary with age and physiological conditions, such as fatigue [28]. For instance, aging may alter how caffeine influences circadian rhythms and overall physiological function. Thus, we hypothesized that caffeine can induce behavioral changes as well as affect circadian clock gene expression. These effects of caffeine appear to be exhibited differently with aging (Fig. 1). To explore these effects, we studied caffeine's impact on circadian rhythms and behavior in a murine model, including how caffeine interacts with physiological fatigue and adenosine. In this study, male C57BL/6 mice were divided into control, vehicle-treated, caffeine-treated, and caffeine with adenosine groups to assess caffeine's interaction with adenosine. Behavioral tests showed that caffeine reduced weight gain and improved motor coordination. Molecular analysis revealed significant changes in circadian clock gene expression, indicating caffeine's potential to disrupt circadian regulation. This research provides insights into caffeine's multifaceted effects on physiology and behavior, especially regarding circadian rhythm modulation.

## 2. Materials and Methods

### *2.1 Animal Experimental Design*

In this study, we used only male C57BL/6 mice with ARRIVE 2.0 guidelines, due to documented differences in the effects of coffee and caffeine intake between male and female rodents and humans (**Supplementary Table 1**) [29,30]. Sample size was determined using power analysis, with a significance level (α) of 0.05 and statistical power (1–β) of 0.80. Clear inclusion and exclusion criteria were established before data collection. Inclusion criteria comprised healthy mice within the specified age ranges and without any observable abnormalities. Exclusion criteria included signs of illness, injury, abnormal baseline behavior, or technical issues during treatment or data acquisition. No animals meeting predefined criteria were excluded unless specified in the Results section, where final analyzed sample sizes (n) are reported. in the Results. Animals were randomized to treatment groups using an Excel-based randomizer. Both the experimenter and the person analyzing

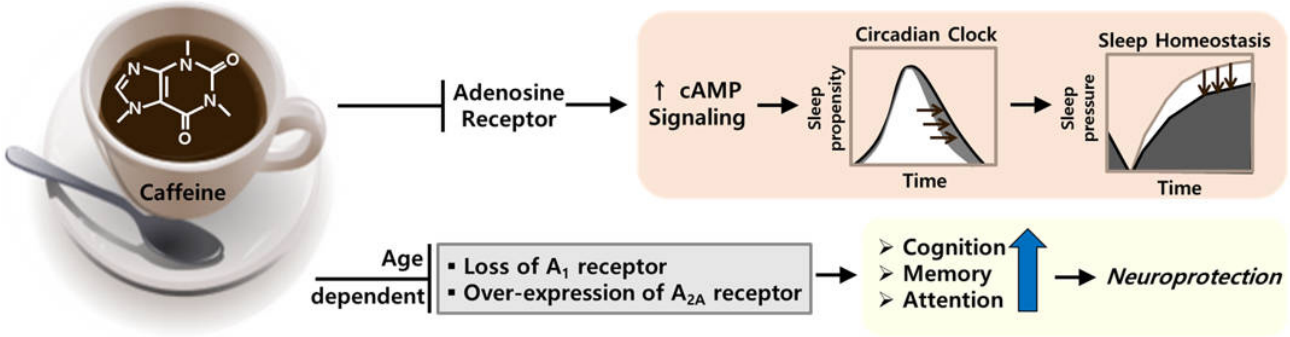


**Fig. 1. Hypothetical scheme of the study**. Caffeine can induce behavioral changes, as well as affect circadian clock gene expression, which can depend on aging.

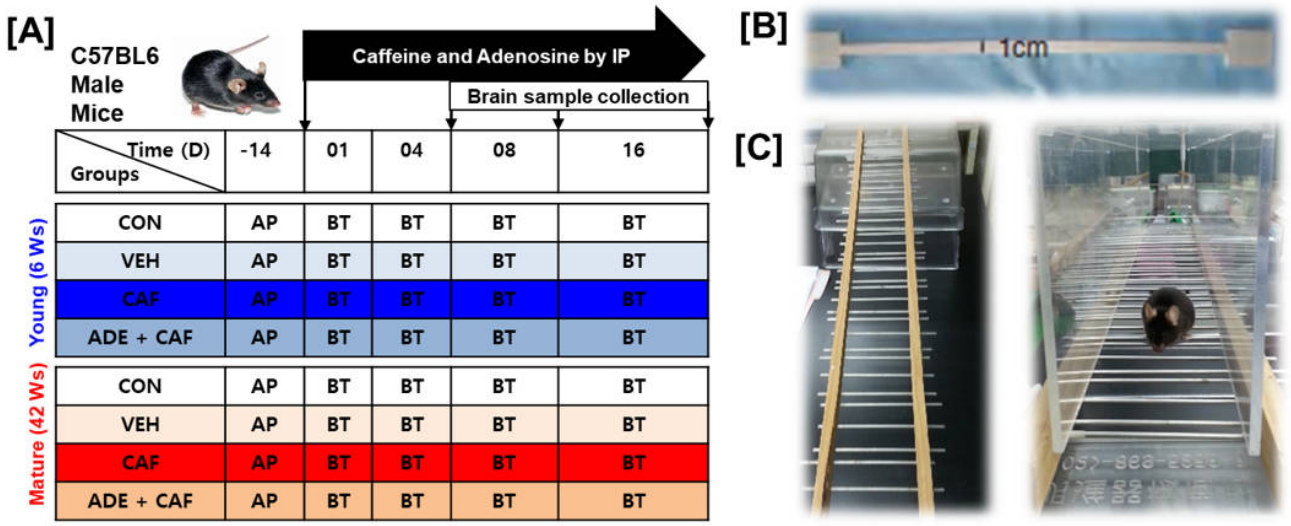


**Fig. 2. Experimental design and behavioral tests in mice**. (A) Study experimental design of young and mature mice were divided into control and three treatment groups, (B) beam structure for beam walk test, and (C) grid ladder to measure of foot faults number. CON, control; VEH, vehicle; CAF, caffeine; ADE, adenosine; IP, intraperitoneal; AP, acclimatization period; BT, behavioral test; D, days; Ws, weeks.

the data were fully blinded to treatment allocation throughout the study. Both the experimenter and the data analyst were fully blinded to treatment allocation throughout the study. To evaluate the age-dependent effects of caffeine, we selected two age groups: young mice (1 month 2 weeks) and mature mice (10 months 2 weeks), both purchased from Dae-Han NioLink (Hyochang Science, Daegu, Republic of Korea). Four mice were housed per cage under a 12:00 hr light/12:00 hr dark cycle at 30% humidity in an atmospheric animal house. During the 2-week acclimatization period, the mice had free access to standard rodent chow (Hyochang Science) and tap water. After acclimatization, the mice were randomly divided into a control group (Con; n = 8) and three experimental groups: vehicle (Veh; n = 8), caffeine (Caf; n = 8), and caffeine with adenosine (Caf+Ade; n = 8) (Fig. 2A). The combination of caffeine and adenosine treatments was designed to investigate caffeine's blocking effect on adenosine's activation and inhibition of A1 and A2A receptors. Molecular grade caffeine (C0750-5G, Sigma-Aldrich Korea, Seoul, Republic of Korea) was prepared in methanol, and adenosine (A9251-1G, Sigma-Aldrich) was prepared in Tris HCl/$H_2O$ (5 mM). Caffeine was administered at a dose of 30 mg/kg intraperitoneally [31,32], and adenosine was injected at a dose of 10 mg/kg intraperitoneally [33], for 16 consecutive days, between 12:00–13:00 to avoid circadian variability. During the treatment period, the same free access to control diet and tap water was supplied to all experimental groups, including the control group. Body weight was measured daily, and behavioral test were performed on day 1, 4, 8, and 16. The mice were anesthetized and decapitated, and brain samples were collected on days 4, 8, and 16 (last day of experiment termination; Fig. 2A). Anesthesia was conducted by using 3% isoflurane (657801261, Hana Pharm, Seoul, Republic of Korea), and euthanasia was confirmed by collecting blood directly from the heart. Isoflurane has mod-

ulatory effects on adenosine signaling in the brain, but not as a primary mechanism, and data do not support its action through pathways involving adenosine [34,35]. Only male C57BL/6 mice were used in this study to minimize variability arising from hormonal fluctuations across the estrous cycle in females, which can significantly influence caffeine metabolism, locomotor responses, and neurophysiological outcomes in young and mature mice.

### *2.2 Behavioral Test*

Behavioral tests were conducted on day 1, day 4, day 8, and day 16 of treatment. Before caffeine administration, mice underwent forced treadmill exercise to induce physiological fatigue, then balance and coordinated locomotion were quantified with a beam walk test and a foot fault test (Fig. 2B,C). For the beam assay, mice traversed a wooden beam 80 cm long, 1 cm wide, mounted 60 cm above the surface; the top surface was lightly roughened to prevent slipping, start/finish lines were marked 5 cm from the ends, and the beam was hand-marked every 10 cm to score distance. Mice were acclimated to the behavioral room for 30 min on test day and trained 1 day prior under identical conditions (3 trials per mouse, 10 min inter-trial interval). On test day, each mouse performed 3 trials (10 min inter-trial interval); at trial start the mouse was placed immediately before the start line, and the trial ended upon crossing the finish line or at 60 s, whichever occurred first. Two synchronized cameras recorded every trial: a rear view to score hind foot slips (defined as a hind paw losing contact with the top surface and dropping below the beam edge) and a lateral view to quantify latency to initiate (time from placement to crossing the start line), traverse time (start-to-finish), distance traveled (number of 10-cm intervals crossed), and falls; apparatus were cleaned with 70% ethanol between animals under uniform illumination. For the foot fault test (grid-walking variant), mice traversed an elevated wire grid platform 40 × 40 cm with 12 × 12 mm openings (1.5 mm wire), elevated 40 cm above the floor; each mouse completed 3 trials (10 min inter-trial interval), each 60 sec or until crossing from start to finish. A foot fault was defined as any fore or hind paw slipping through the grid below the mesh plane; total faults and total steps were counted from video to compute a fault rate (faults/steps), alongside latency to initiate, traverse time, distance covered (grid squares crossed) and falls. If a ladder-rung variant was used, the walkway comprised a 100 cm ladder set at 15° tilt with 2 mm-diameter rungs spaced 10 mm (fixed pattern), elevated 40 cm; a foot fault was defined as a miss or slip below the rung plane, with the same trial structure and scoring (faults/steps, time, distance, falls).

### *2.3 Tissue Sample Collection*

After the experimental period, mice were sacrificed according to the procedures described earlier. The brain samples were collected for gene expression and protein quantification. For the gene expression, the left half-brain samples were collected in pre-labeled, pre-chilled EP-tubes (filled with TRIzol reagent; 15596026, Invitrogen, Carlsbad, CA, USA) and snap-froze in $LN_2$, while for protein quantification, the left half-brain samples were collected in pre-labeled, pre-chilled EP tubes and snap-froze in $LN_2$. The samples were immediately stored at –80 ºC for further analysis.

### *2.4 Gene Expression by Reverse Transcriptase-Polymerase Chain Reaction*

Frozen brain tissues were used to extract and quantify the total RNA using TRIzol reagent, treated with the RNase-free DNase, and used for cDNA synthesis. The cDNA is synthesized by the reverse transcriptase enzyme (Invitrogen). *Per2*, *Cry1*, *Cry2*, and *Clock* were used as target genes, and *β-actin* was used as a housekeeping gene (control). Mice lacking these genes are related to impaired coordination, more slips/falls in balance, and increased foot faults [36,37]. Endpoint reverse transcriptase polymerase chain reaction (RT-PCR) was performed using cDNA generated from high-quality RNA as template (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Primer sets (Table 1) were designed to yield amplicons of 100–250 bp with balanced GC content and matched melting temperatures. Gradient PCR was initially employed across a 55–68 °C range to determine optimal annealing conditions, selecting the temperature that produced a single, specific band of the expected size without nonspecific amplification. Following temperature optimization, various primer concentration gradients (200–500 nM) were tested to further refine amplification efficiency and specificity. The finalized amplification protocol consisted of one cycle of denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for all genes except *β-actin* (60 °C, 1 min), and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplified DNA fragments were separated on 2% agarose gel and visualized with 0.5 µg/mL ethidium bromide using a UV illuminator (Model AXYGDAX001, Corning Incorporated, Corning, NY, USA). Negative controls (no-template and –RT) were included to confirm specificity, and band identity was verified by expected size. In addition, the stability of internal reference genes was evaluated to ensure reliable normalization across experimental groups. *β-actin* was specifically assessed in mice of different age groups, and no differences in its stability were observed between young and mature animals. This verification confirmed that *β-actin* was an appropriate internal control for subsequent endpoint RT-PCR analyses.

### *2.5 Protein Quantification by Western Blot*

Frozen stored brain tissue was homogenized with liquid nitrogen and RIPA lysis buffer (PI89900, Thermo Fisher Scientific) to extract the total protein. The homogenate

**Table 1. Details of primer sequence used for RT-PCR.**

| Gene | Primer sequence (5′-3′) | Product length (bp) | GenBank accession No. |
|---|---|---|---|
| *Bmal1* | F: AAGGGCCACTGTAGTTGCTG<br>R: GGCACGTGAAAGAAAAGCAC | 147 | NC_000073.7 |
| *Per2* | F: GGC TGT GTC CCT GGT TTC TG<br>R: CCA CAA ACT TGG CAT CAC TGA | 101 | NM_031678.1 |
| *Cry1* | F: CTT CCA ACG TGG GCA TCA AC<br>R: CCG AAT CAC AAA CAG ACG AGA A | 101 | NM_007771.3 |
| *Cry2* | F: ACA CAG GCC CCA GAG CAC TA<br>R: TCT AGC CCG CTT GGT CAG TT | 101 | NM_009963.4 |
| *Clock* | F: AAA TAT TCA TCG GCA AGA AG<br>R: CAG GGT TTG ATT GCT GCA AA | 101 | NM_007715.6 |
| *β-actin* | F: TAA AGA CCT CTA TGC CAA CAC AGT<br>R: CAC GAT GGA GGG GCC GGA CTC ATC | 241 | NM_031144.2 |

RT-PCR, reverse transcriptase-polymerase chain reaction; *Per*, Period Circadian Regulator; *Cry*, Cryptochrome Circadian Regulator; *Clock*, Circadian Locomotor Output Cycles Kaput.

was then centrifuged at 12,000 ×g for 15 min, and the supernatant was collected. Protein concentration was quantified using the BCA protein assay kit (23225, Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. Protein samples were prepared using sample buffer (#1610737, Bio-Rad, Hercules, CA, USA), and a total of 30 µg/well proteins were loaded to assess adenosine receptor protein quantification. After gel electrophoresis, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (IPVH00010, Merck Millipore, Burlington, MA, USA) using a wet transfer system with transfer buffer. Once the transfer finished, it was then blocked with tris-buffered saline with Tween-20 buffer (TBST, 9997, Cell Signaling Technology, Danvers, MA, USA) containing 5% non-fat dry milk for 2 hr and then washed by using TBST washing solution, and the membrane was incubated for overnight at 4 °C in primary antibodies: anti adenosine receptor 1 (A1R) (1:500, Abcam, Cambridge, UK, Cat# ab82477), anti-adenosine receptor 2A (A2AR) (1:5000, Thermo Fisher Scientific, Cat# PA1-042), and anti GAPDH (1:1000 Cell Signaling Technology Cat# 2118). The following day, after washing with TBST, membranes were incubated using horseradish peroxidase-labeled secondary antibodies (1:5000) for 60 min. Band intensity was measured using a Bio-Rad Gel-Doc imaging system (Bio-Rad, Hercules, CA, USA).

### *2.6 Cell Line Experimental Design*

To validate the effect of caffeine on circadian clock gene expression in the *in vivo* study, we further performed tests to verify the protein and gene expression of the circadian clock gene in the *in vitro* study using mouse Neuro 2a (N2a) cells (Fig. 3). N2a cells were purchased from American Type Culture Collection (CCL-131, ATCC, Manassas, VA, USA) and cultured in complete α-Minimum Essential Medium (12571-048, α-MEM; Gibco, Thermo Fisher Scientific) media supplemented with 3% Fetal Bovine Serum (FBS; 16000-044, Gibco, Thermo Fisher Scientific) in a 37 ºC incubator with 5% $CO_2$ according to ATCC recommendations. Cells were maintained at a density of $5 \times 10^5$ cells per dish. N2a cells passage number 27 stocked, and used for experiments. The N2a cells have been used earlier for studying circadian clock gene expressions by Roy et al, 2022 [38]. Before the time-dependent effect of caffeine, cell viability and morphology of the cells were determined with different concentrations of caffeine. For that, N2a cells were seeded in a 96-well plate ($3 \times 10^4$ cells/100 µL/well) in α-MEM media supplemented with 3% FBS in a 37 ºC incubator with 5% $CO_2$ for 24 hr [39]. After 24 hr of incubation, the old media were replaced with α-MEM media supplemented with 1% FBS and different concentrations of caffeine (0.5, 1.5, and 3 mM caffeine, along with 5 and 10 mM) for 24 hr, in accordance with previous studies [40,41]. To prevent the high progression of the cell cycle, a low concentration of FBS (1%) is used to maintain the normal condition of the cells. This was important to ensure that changes in expression over time were regulated by the circadian clock gene, not by the cell cycle. After a 24 hr incubation period, the treated media were aspirated, and trypan blue (T6146-5G, Sigma-Aldrich) staining and MTT assay were performed using the CyQUANT MTT kit (V13154, Thermo Fisher Scientific) according to the manufacturer's instructions (Fig. 3A). The morphology of the cells was observed by light microscopy, and the caffeine concentration of 1.5 mM was selected based on cell viability for molecular mechanism experiments. For circadian clock gene expression, the N2a cells were seeded at a density of $3 \times 10^5$ cells/dish in 100 mm petri dishes and grown at 37 ºC in a 5% $CO_2$ atmosphere incubator. Cells are allowed to grow to 70–80% of confluence. For time laps (0 h, 4 h, 8 h, 12 h,16 h, 20 h and 24 h) expression of circadian clock genes was assessed by RT-PCR (Fig. 3B). For circadian clock synchronization (i.e., to entrain the cell population to the same circadian phase), cells were treated with 200 nM dexametha-

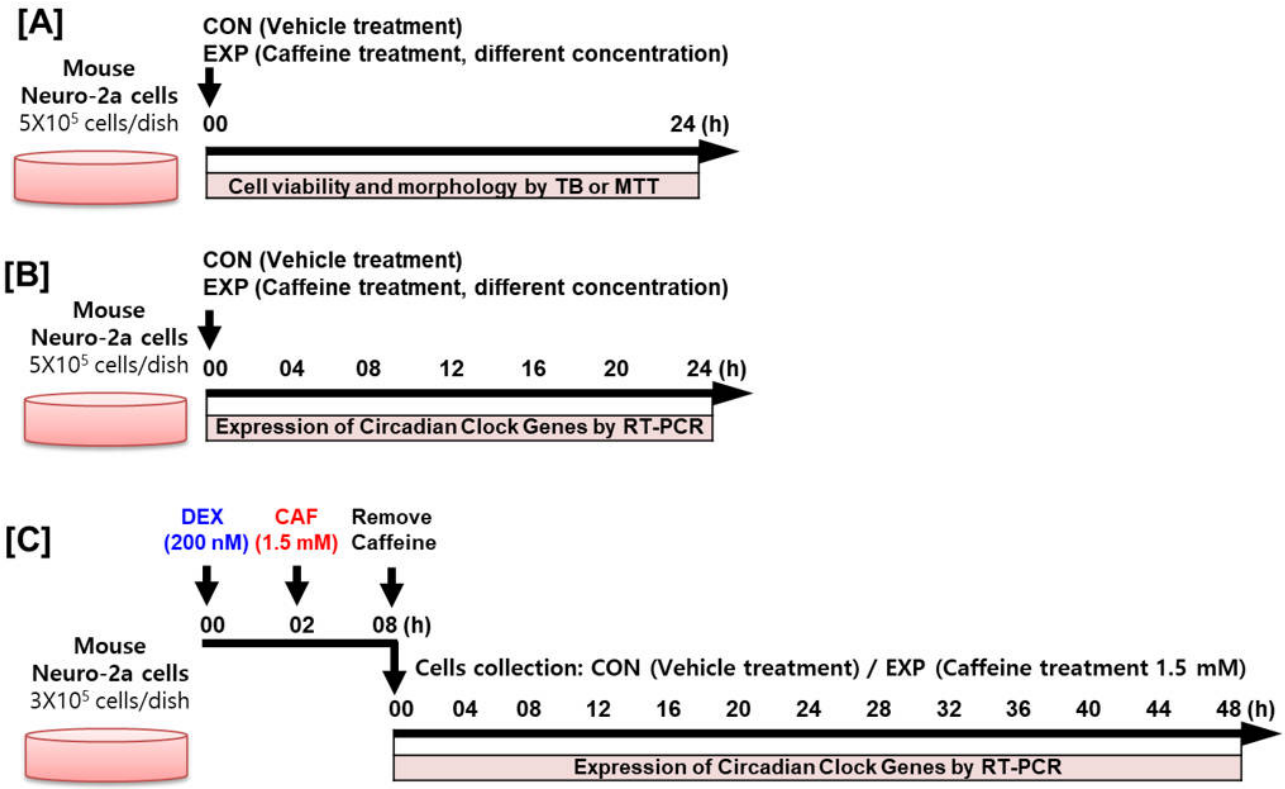


**Fig. 3. Cell line experiment design in N2a cell line**. (A) Effect of caffeine on cell viability, (B) effect of caffeine on circadian clock gene expression, and (C) effect of caffeine on circadian clock gene expression of pretreated *DEX*. EXP, Experimental; DEX, Dexamethasone; TB, Trypan blue; N2a, Neuro 2a.

sone (D4902, Sigma-Aldrich) for 2 hr with α-MEM media containing 1% FBS. Subsequently, dexamethasone-treated media were replaced by α-MEM media supplemented with 1% FBS and 1.5 mM caffeine for 6 hr. Finally, the caffeine-treated medium was replaced by 1% FBS containing α-MEM media. Cells were collected every 4 hr for 48 hr under a cool environment, and total RNA was extracted for RT-PCR as mentioned above (Fig. 3C). The N2a cell line was authenticated by short tandem repeat (STR) profiling performed by ATCC using the Promega PowerPlex® 16 HS System. The STR profile matched the reference profile for N2a in the ATCC and DSMZ databases, confirming its identity and ruling out cross-contamination with other common cell lines. Mycoplasma contamination was tested monthly using the MycoAlert™ Mycoplasma Detection Kit (LT07-118, Lonza, Basel, Switzerland); the ratio of Reading B/Reading A was consistently <0.9, indicating the culture was mycoplasma-negative throughout the study.

### *2.7 Statistical Analysis*

Data were plotted using observed values of biological replicate experiments, and all statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 22.0 (IBM, New York, NY, USA). Results are presented as mean ± SD. Statistical analyses were selected according to the experimental design and data structure. One-way ANOVA was used for experiments involving a single independent factor, such as cell-based assays and *in vitro* gene expression studies. Two-way ANOVA was applied to molecular data sets (gene and protein expression) to assess the main effects of age and treatment, as well as their interaction, and analyses were performed separately for each time point when applicable. Behavioral data, which involved repeated testing of the same animals across multiple trials, were analyzed using repeated-measures ANOVA to account for within-subject variability. When significant omnibus effects were detected, Tukey's post hoc multiple comparison test was used to determine specific group differences. A $p$-value $\leq 0.05$ was considered statistically significant.

## 3. Results

### *3.1 Age-Dependent Modulation of Body Weight and Feeding Behavior by Caffeine and Adenosine in Mice*

To examine the physiological effects of caffeine, mice were divided into four groups—vehicle, caffeine, caffeine + adenosine, and a control group—and maintained on their respective treatments for 16 days. Behavioral tests were conducted on day 1, day 4, day 8, and day 16 of treatment. On day 1 of injection, the mean difference in body weight

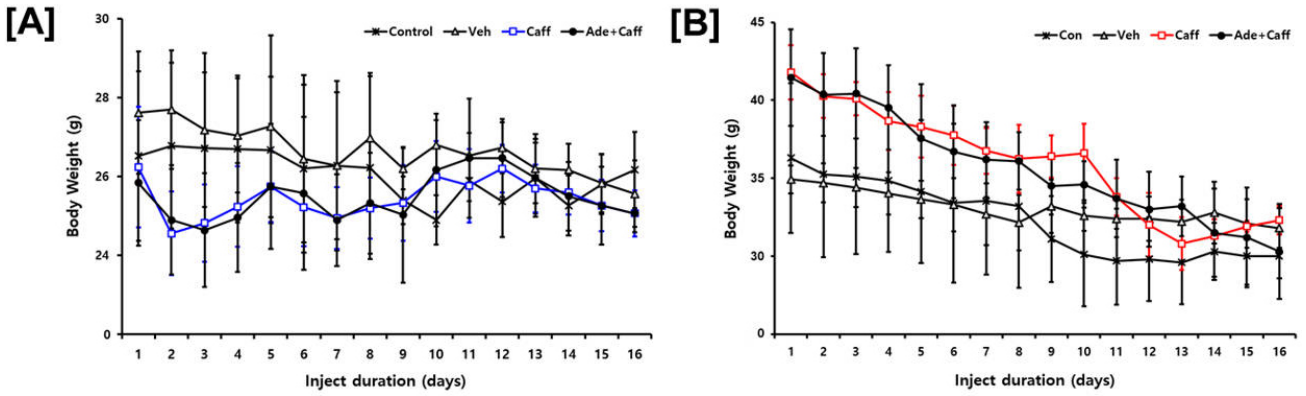


**Fig. 4. Effect of caffeine on physiological change in young and mature mice**. (A) Changes in body weight of young mice and (B) change in body weight of mature mice. Body weight was monitored longitudinally throughout the experimental period. Data are presented as mean ± SD (n = 8 animals per group). Statistical analysis was performed using two-way ANOVA with age and treatment as between-subject factors. No statistically significant differences were observed between groups at day 1 or day 16. The average difference in body weight of young mice at day 1 of injection between groups was ~2 grams, whereas the average difference in body weight of mature mice at day 1 of injection between groups was ~ 6 grams. On the other hand, the average difference in body weight of young mice at day 16 of injection between groups was reduced to ~2 grams, the trend was similar as indicated by average difference in body weight of mature mice at day 16 of injection between groups, which was reduced to ~1 gram. There was no statistically significant difference between various groups either at day 1 or day 16. Eight animals were used in each group.

between groups was about 2 g for young mice and about 6 g for mature mice, reflecting natural age-related weight differences. By day 16th, young mice showed no significant change in body weight relative to the control (Fig. 4A), whereas mature mice exhibited a slight decrease followed by a modest increase starting on day 13 compared with the control (Fig. 4B). Comparison of the initial (day 0) and final (day 16) body weights revealed a trend toward reduced weight gain in caffeine-treated mice versus control, though this difference did not reach statistical significance. Nonetheless, a difference between groups remained apparent. These data suggest that caffeine may inhibit food intake in both young and mature mice, even though no statistically significant differences were observed at day 1 or day 16.

### *3.2 Chronic Caffeine Administration Enhances Beam-Walk Performance and Reduces Foot-Faults in Young and Aged Mice*

Three behavioral tests were performed with the young and mature mice after caffeine treatment on days 1, 4, 8, and 16 of caffeine treatment. The beam walk test concerning the time and error, and the foot fault test concerning the misplacement of fore and hind foot were performed. The average time to cross the beam and the number of foot slip errors after caffeine injection were decreased in the caffeine group in both young and mature mice compared with their control groups (Fig. 5A,B). In addition, the foot fault, which was defined as the misplacement of the fore or hind foot, was reduced after caffeine treatment compared with the control (Fig. 5C). Beam walk test results showed a trend but remained statistically insignificant. Collectively, these behavioral tests suggested that changes in balance ability and improvement in coordination of both young and mature mice groups during 16 days of caffeine and adenosine treatment reflected an improved locomotor stimulatory effect.

### *3.3 Caffeine Modulates Circadian Rhythm Gene Expression in Young and Mature Mice*

To investigate the molecular effects of caffeine on circadian regulation, we analyzed the expression of four core clock genes (*Per2*, *Cry1*, *Cry2*, and *Clock*) in the brains of young and aged mice following 4, 8, and 16 days of treatment. RT-PCR analysis revealed no significant changes in gene expression at days 4 and 8 in either age group. However, by day 16, caffeine treatment induced significant alterations in all four genes, with distinct patterns observed between young and mature mice (Fig. 6A,B, the original western blot images can be found in the **Supplementary Materials-WB-Dataset**).

In young mice, caffeine (G2) significantly upregulated *Per2* ($p < 0.05$ vs. Control), *Cry1* ($p < 0.05$ vs. Control), and *Clock* ($p < 0.05$ vs. Control), while *Cry2* expression remained unchanged. The G3 (~relative abundance 87.35) group (caffeine + adenosine) showed attenuated expression compared to G2 (~relative abundance 97.22), suggesting adenosine may counteract caffeine's stimulatory effect ($p < 0.05$ vs. G1). In aged mice, G2 led to a downregulation of *Per2* and *Cry1* ($p < 0.05$ vs. Control), while *Cry2* and *Clock* were significantly upregulated ($p < 0.05$ vs. Control). Notably, intergroup comparisons revealed age-dependent differences in gene responsiveness: *Per2* and *Cry1* expression

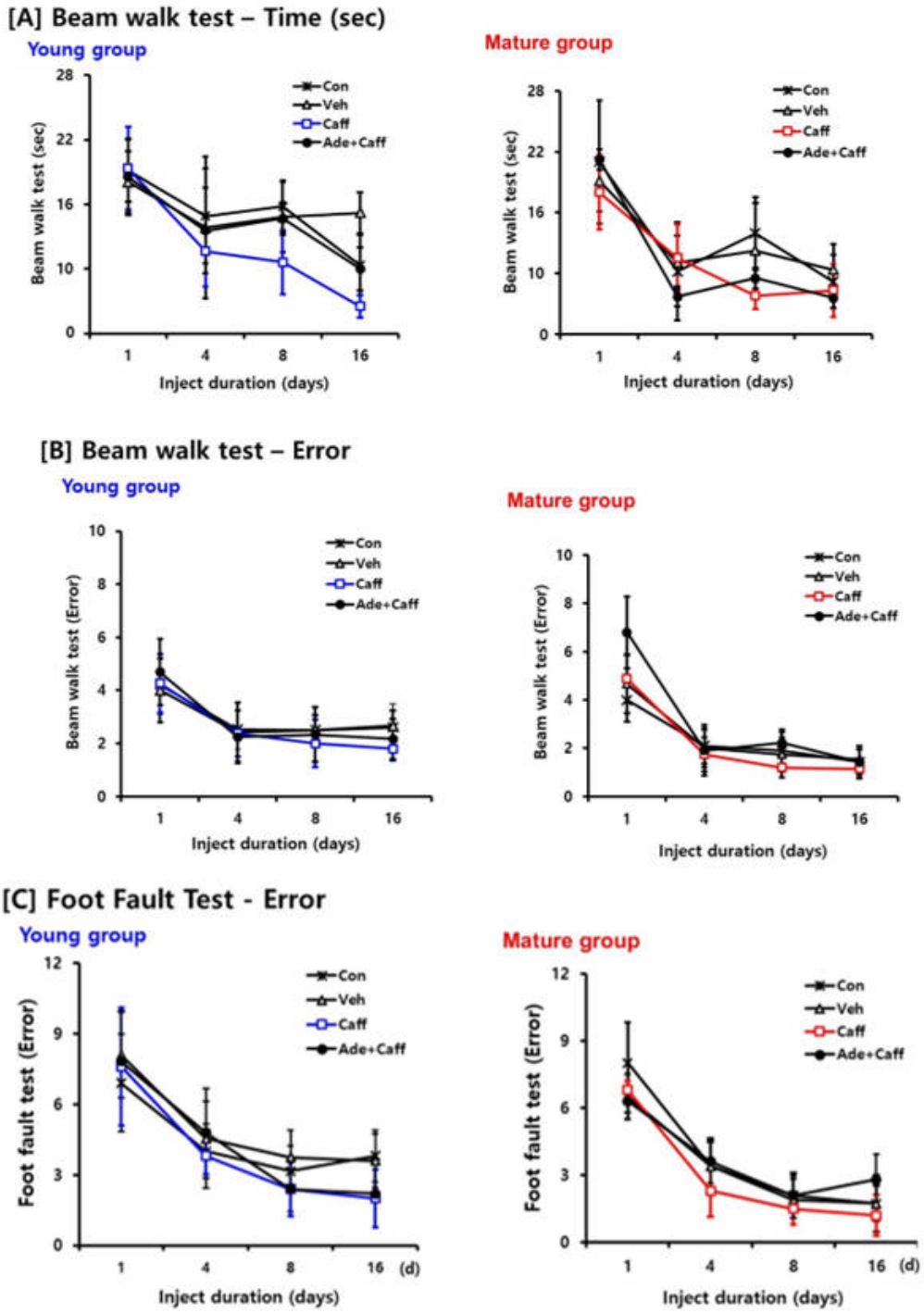


**Fig. 5. Effect of caffeine on behavioral tests in young and mature mice**. (A) Averaged time to cross the beam was recorded in each run of the test, (B) slipping error was recorded in each run of the test, and (C) misplacement of paws were decreased in foot fault test in both young and mature mice groups. Behavioral performance was assessed repeatedly within the same animals across multiple trials. Data are presented as mean ± SD (n = 8 animals per group). Statistical analysis was performed using repeated-measures ANOVA with trial as the within-subject factor and age and treatment as between-subject factors, followed by Tukey's post-hoc multiple comparison test.

were significantly lower in mature mice compared to young mice after 16 days ($p < 0.05$ vs. Young), indicating a possible age-related decline in caffeine-mediated circadian stimulation.

These findings suggest that caffeine exerts gene-specific and age-dependent regulatory effects on the circadian clock. The upregulation of *Per2* and *Cry1* in young mice may reflect enhanced circadian amplitude and behav-

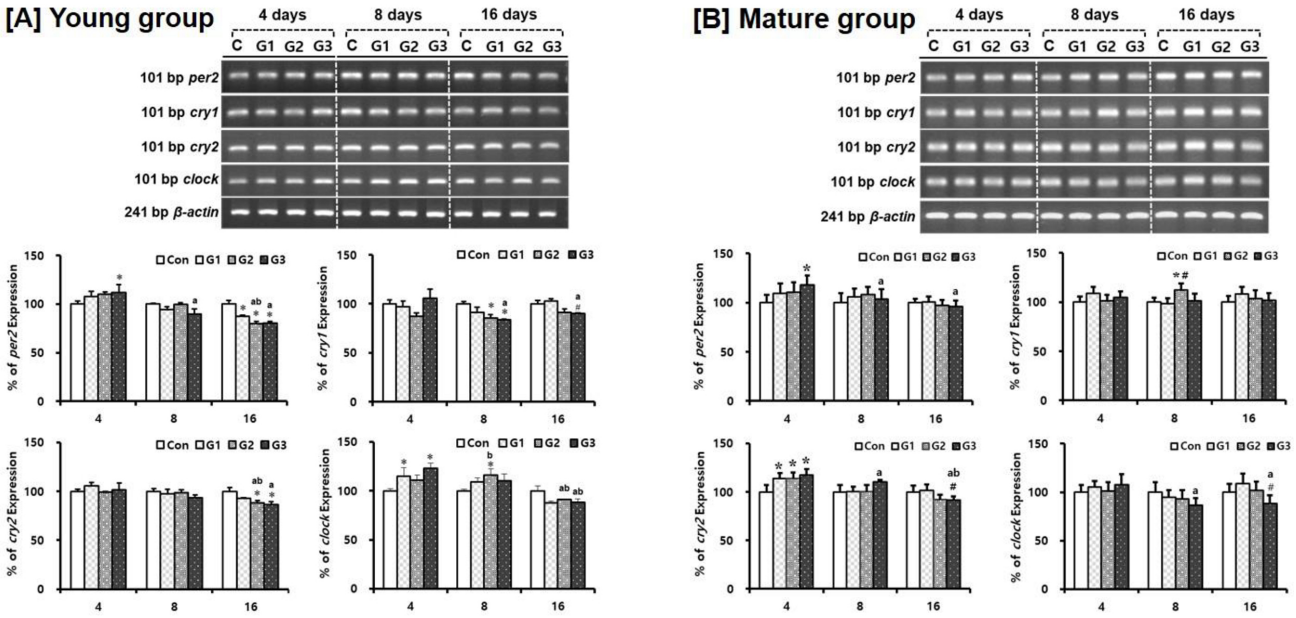


**Fig. 6. Effect of caffeine on circadian rhythm gene mRNA expression in young and mature mice brain tissue by RT-PCR**. (A) Comparison of circadian clock genes expression levels after caffeine treatment in young groups at day 4, 8 and 16. (B) Comparison of circadian clock genes expression levels after caffeine treatment in mature groups at 4, 8, and 16 days. Gene expression was quantified by RT-PCR and β-actin. Data are presented as mean ± SD (n = 3 animals per group). Statistical analysis was performed separately for each gene and time point using two-way ANOVA with age and treatment as factors, followed by Tukey's post-hoc test. *$p < 0.05$ vs control; #$p < 0.05$ vs G1. Con, control; G1, vehicle; G2, caffeine; G3, caffeine + adenosine. Different lowercase letters (a, b, ab) indicate statistically significant differences among treatment groups within the same time point ($p < 0.05$), where groups sharing at least one letter are not significantly different.

ioral activation, consistent with caffeine's known stimulant properties. Conversely, the attenuated or reversed expression in aged mice may indicate altered sensitivity of the aging circadian system to caffeine, potentially linked to changes in adenosine receptor signaling or neuroplasticity.

### *3.4 Long-Term Caffeine Administration Modifies Adenosine Receptors, A1R and A2AR, Protein Levels in the Brain of Young and Mature Mice*

The protein quantification of adenosine receptors (A1R and A2AR) was done by western blot in young and mature mice at days 4, 8, and 16 in the brain sample. The protein quantification of adenosine receptors (A1R and A2AR) at day 4 (Fig. 7A), and 8 showed no difference (Fig. 7B), while the level at the day 16 caffeine-treated brain showed some significant difference between the groups of young and mature mice (Fig. 7C). These results reveal that, after a long time, caffeine administration impacts the level of adenosine receptor protein in both young and mature mice, regardless of age (The original western blot images can be found in the **Supplementary Materials-WB-Dataset**).

### *3.5 Age-Dependent Effects of Caffeine on Circadian-Clock Genes and Adenosine-Receptor Proteins in the Mouse Brain*

We examined how chronic caffeine treatment modulates the expression of core circadian clock genes (*Per2*, *Cry1*, *Cry2*, *and Clock*) and adenosine receptor proteins (A1R, A2AR) in young and mature mice. Across 4, 8, and 16 days of treatment, clock gene transcripts remained unchanged in the young cohort. In contrast, mature mice exhibited a significant downregulation of *Cry1* at day 16 compared to young mice ($p < 0.05$; Fig. 8), suggesting age-specific sensitivity to caffeine. These findings indicate that aging alters circadian gene responsiveness under chronic caffeine exposure, potentially contributing to differential behavioral and physiological outcomes across age groups.

### *3.6 Caffeine Modulates Cell Viability, Morphology, and Circadian Gene Expression in N2a Neuroblastoma Cells*

Cell viability was evaluated by trypan-blue exclusion and MTT assays. Caffeine had no significant effect on viability at 0.5, 1.5, and 3 mM caffeine, along with 5 and 10 mM produced marked decreases compared to the control (**Supplementary Fig. 1A**). Morphologically, cells exposed to high doses (5 and 10 mM) displayed rounded bodies and shortened dendrites (5 and 10 mM) (**Supplementary Fig.**

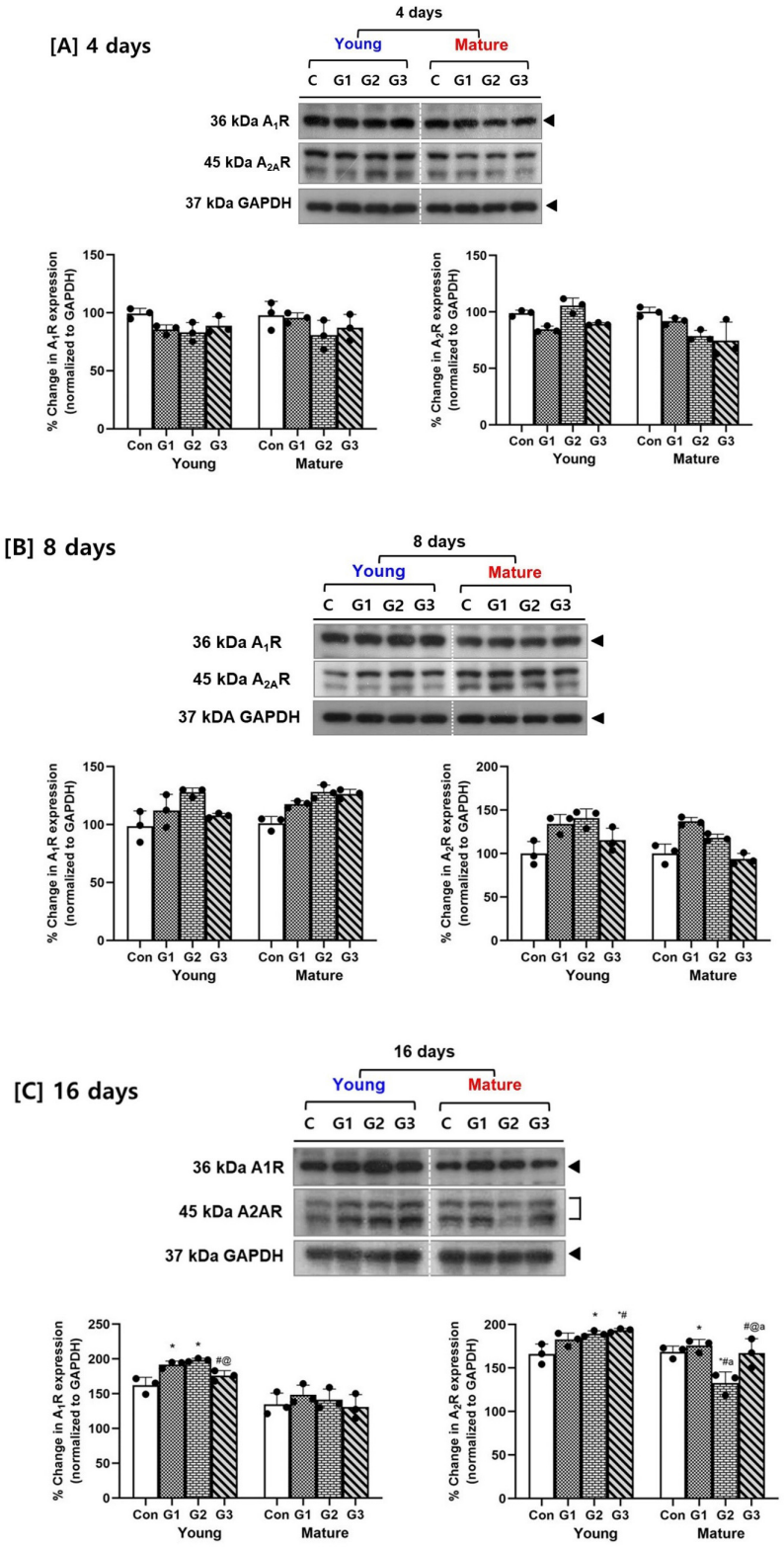


**Fig. 7. Effect of caffeine on adenosine receptor protein levels by western blot**. (A) Comparison between young and mature after caffeine treatments at day 4, (B) comparison between young and mature after caffeine treatments at day 8, and (C) comparison between young and mature after caffeine treatments at day 16. Protein expression levels were normalized to GAPDH and expressed relative to control. Data are presented as mean ± SD (n = 3 animals per group). Statistical analysis was performed separately at each time point using two-way ANOVA with age and treatment as factors, followed by Tukey's post-hoc multiple comparison test. *$p < 0.05$ vs control; #$p < 0.05$ vs G1; @$p < 0.05$ vs G2; $^{a}p < 0.05$ vs young groups. C, control; G1, vehicle; G2, caffeine; G3, caffeine + adenosine; ARs, adenosine receptors (A1R and A2AR).

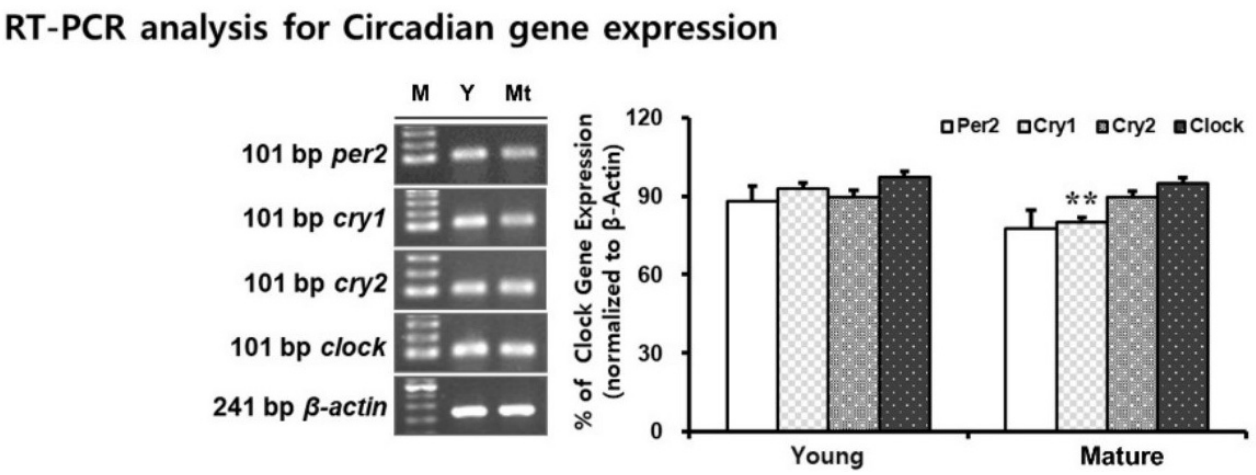


**Fig. 8. Effect of caffeine on gene and protein differential expression patterns between young and mature mice**. Expression of circadian clock genes, and protein. Data represent mean ± SD (n = 3 animals per group). Statistical analysis was performed using two-way ANOVA with age and treatment as factors, followed by Tukey's post-hoc test for multiple comparisons. $^{**}p < 0.01$ between young and mature groups. M, marker; Y, young; Mt, mature.

**1B**) (Fig. 9A). The original western blot images can be found in the **Supplementary Materials-WB-Dataset**.

*3.7 Caffeine Modulates Circadian Clock Gene Expression in N2a Cells*

To evaluate the molecular impact of caffeine on circadian regulation, we performed two independent experiments using N2a neuroblastoma cells. In the first experiment, cells were treated with 1.5 mM caffeine for 24 hr, and gene expression was assessed at multiple time points. In the second experiment, cells were first synchronized with dexamethasone and then treated with caffeine; samples were harvested every 4 hours over a 48-hour period to capture circadian oscillations.

In the caffeine-only condition, expression levels of core clock genes were significantly downregulated at all measured time points compared to vehicle-treated controls ($p < 0.05$; Fig. 9B). This suggests that caffeine alone may suppress basal circadian gene expression in unsynchronized cells. In contrast, when cells were pre-synchronized with dexamethasone, caffeine treatment led to a transient upregulation of all five genes, peaking at 24 hr, followed by a significant decline in expression from 28 to 48 hr ($p < 0.05$; Fig. 9C). These biphasic dynamics indicate that caffeine may enhance circadian gene amplitude in synchronized cells before inducing a dampening effect.

Together, these findings demonstrate that caffeine exerts context-dependent regulatory effects on circadian clock gene expression in N2a cells. The initial upregulation in synchronized cells may reflect enhanced transcriptional activation, while the subsequent decline suggests feedback inhibition or desynchronization. These results support the hypothesis that caffeine can modulate circadian rhythm machinery, with implications for time-of-day–dependent pharmacological responsiveness.

## 4. Discussion

In this study, we examined the effect of caffeine on behavior and circadian rhythms in a male C57BL/6 murine model, focusing on how caffeine interacts with clock genes and adenosine receptor expression in young and mature mice. Previous studies have demonstrated clear sex differences in caffeine-induced behavioral sensitization and neurochemical changes in mice [42], rats [43], and human [44]. To minimize biological variability and isolate age-dependent effects, our study focused exclusively on male mice, allowing clearer interpretation of age-related differences in caffeine responsiveness. The experimental design included one control group and three treatment groups (vehicle-treated, caffeine-treated, and caffeine plus adenosine-treated). The combined treatment of caffeine and adenosine was deliberately chosen to investigate potential functional antagonism at adenosine receptors under conditions of elevated extracellular adenosine, a scenario relevant to physiological states such as brain ischemia, sleep deprivation, or intense neuronal activity, where adenosine levels rise markedly. The inclusion of the caffeine + adenosine group was deliberate and mechanistically motivated, aiming to probe functional antagonism at adenosine receptors under conditions of elevated extracellular adenosine. Such conditions are physiologically relevant in states including brain ischemia, sleep deprivation, and heightened neuronal activity, where adenosine accumulation acts as a homeostatic inhibitory signal. Although caffeine is a non-selective A1/A2A receptor antagonist, co-administration with exogenous adenosine allows us to test whether high local concentrations of the endogenous agonist can overcome or reverse caffeine's effects on neuronal excitability, synaptic transmission, and behavioral outcomes, thereby clarifying the dy-

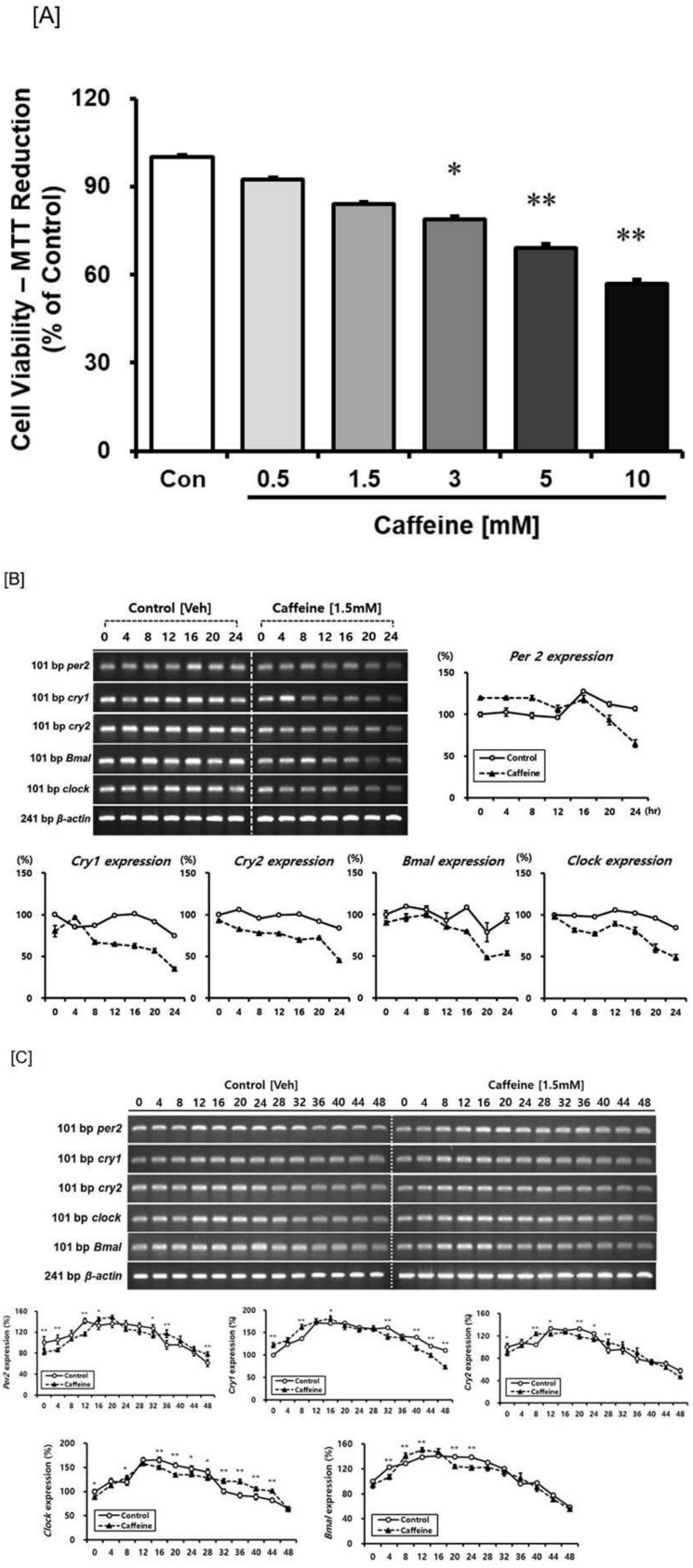


**Fig. 9. Effect of caffeine on cell viability and clock gene expression in N2a cells**. (A) Cell viability following caffeine treatment for 24 hr. (B) Circadian clock genes expression following caffeine treatment for 24 hr. (C) Circadian clock genes expression following dexamethasone and caffeine treatment for 48 hr. $*p < 0.05$, $**p < 0.01$ vs. Control. ANOVA followed by Tukey's post-hoc analysis.

namic range and competitive nature of this interaction *in vivo* [2,45]. To simulate fatigue, mice underwent forced treadmill exercise prior to caffeine administration. This intervention mimics real-world conditions under which caffeine is frequently consumed and increases extracellular adenosine levels, thereby strengthening interpretation of receptor-mediated effects. After 16 days of treatment, caffeine revealed a trend toward reduced weight gain in both young and mature mice, suggesting that it consistently inhibits food intake across age groups. This uniform reduction in body weight highlights caffeine's broad impact on metabolic processes. Behavioral testing demonstrated that caffeine improved motor coordination, while molecular analyses revealed significant changes in circadian clock gene expression and adenosine receptor protein levels, indicating potential disruption of circadian regulation. Together, these findings indicate that caffeine exerts pleiotropic effects extending beyond locomotor enhancement to include modulation of molecular pathways governing circadian regulation. Overall, these findings suggest that caffeine's physiological effects on energy balance and metabolism are robust regardless of age, providing new insights into its multifaceted influence on physiology and behavior, particularly circadian rhythm modulation.

Caffeine is one of the most widely used psychoactive substances and decreases weight gain [46]. Furthermore, caffeine activates the sympathetic nervous system, leading to an increase in the production of catecholamines such as adrenaline. These catecholamines stimulate lipolysis, the breakdown of fat stores, thereby contributing to reduced weight gain [47]. Additionally, caffeine's effects on metabolic rate are partly mediated through the activation of cAMP and PKA pathways, and elevated levels of cAMP result in increased thermogenesis and metabolic rate, further promoting weight loss [48]. Here, the effect of caffeine on two different age groups of mice: young mice (1 month and 2 weeks) and mature mice (10 months and 2 weeks). We observed a reduction in the body weight gain in both young and mature mouse groups. With aging, the consumption of food decreases due to physiological changes [49]. In addition, aging, considering it is one of the major biological factors, is affected by various factors (food, chemicals, etc.) and affects the normal metabolic and physiological changes [50,51]. After caffeine treatment in young mice, the body weight did not significantly change, while in mature mice, the body weight reduced significantly, which might reflect a reduced consumption of food. Interestingly, while body-weight changes were modest in young mice, mature mice appeared more sensitive to caffeine-induced metabolic modulation. These findings suggest that caffeine's effects on energy balance are preserved across the lifespan, while aging may amplify specific aspects of metabolic responsiveness. The observed decrease in weight gain in both young and mature mice suggests that caffeine's influence on metabolism and appetite regulation is not age-dependent, making it a powerful tool for influencing energy balance across the lifespan. Additionally, the findings indicate that caffeine's effects on appetite suppression and increased energy expenditure could be used in different situations, from controlling weight gain to improving physical performance. This highlights caffeine's potential to influence metabolic rate and appetite, providing a basis for further research into its role in dietary and medical uses. Understanding how caffeine interacts with metabolic processes at a molecular level across different age groups can help optimize its benefits while reducing possible side effects, leading to better weight management and overall health.

As mentioned above, caffeine modulates signaling pathways involving cAMP and PKA [52]. The inhibition of adenosine receptors leads to increased levels of cAMP, which activates PKA [53]. This activation enhances neuronal excitability and synaptic plasticity, thereby supporting improved motor control and cognitive functions related to motor performance. Elevated cAMP levels can also promote neurotransmitter release and increase receptor sensitivity, further contributing to the observed improvements in balance and coordination. In this study, behavioral assessments, including the beam walk and foot fault tests, demonstrated that caffeine treatment improved motor coordination and balance. Specifically, caffeine reduced the number of foot faults, indicating enhanced motor control. In addition, caffeine administration decreased both the average time required to cross the beam and the number of foot slips compared to controls. These findings suggest that caffeine positively influences physical and cognitive functions related to motor performance in both young and mature mice, reinforcing its potential benefits for motor control. However, while a moderate dose of caffeine may improve memory and cognitive function, a high dose could have adverse effects [54]. The young and mature mice treated with the same dose of caffeine (30 mg/kg) showed improvement in balance ability and coordination in behavior tests after caffeine treatments in both young and mature mice.

Caffeine and adenosine modulate circadian rhythms in coordination [55]. Circadian rhythms are an internal biological clock, regulated by clock genes *Bmal1*, Period (*Per1*, *Per2*, *Per3*), and Cryptochrome (*Cry1*, *Cry2*) genes, which synchronize physiological processes and homeostasis [19,20]. Caffeine administration led to differential expression of circadian clock genes, including *Clock*, *Per2*, *Cry1*, and *Cry2*. These changes indicate that caffeine can disrupt circadian rhythms by modulating the molecular components of the circadian clock. This disruption might have the potential for caffeine to interfere with the synchronization of physiological processes regulated by circadian rhythms. Such alterations in circadian gene expression may contribute to the broader impacts of caffeine on sleep, metabolism, and overall well-being. In addition, caffeine impacts circadian rhythms primarily through its antag-

onistic effects on adenosine receptors, specifically A1 and A2A receptors, which are normally involved in feedback mechanisms that regulate clock gene expressions [22,56]. By blocking adenosine receptors, caffeine increases levels of cAMP, which activates PKA and subsequently influences CREB [23]. This cascade alters the expression of circadian clock genes such as *Clock*, *Bmal1*, *Per2*, and *Cry2*, impacting the synchronization of physiological processes [24]. By disrupting these clock proteins, caffeine leads to altered rhythmic expression patterns [14]. Additionally, caffeine-induced changes in clock gene expression can potentially increase the risk of metabolic disorders [25]. Therefore, caffeine's interference with adenosine signaling, cAMP/PKA pathways, and clock protein stability emphasizes its significant influence on circadian rhythms and stresses the importance of strategies to control caffeine intake and its impact on overall health.

Although the N2a cell experiments employed caffeine concentrations that exceed physiological levels, particularly 10 mM, which is widely recognized as cytotoxic, these data provide important contextual support for the *in vivo* findings by defining the extremes of caffeine exposure at the cellular level. In contrast to the mouse experiments, where caffeine acted within a physiological range to modulate behavior, body weight, circadian clock gene expression, and adenosine receptor signaling in an age-dependent manner, supraphysiological caffeine exposure in N2a cells resulted in marked morphological stress, including neurite retraction and cell rounding. This divergence highlights that caffeine's beneficial effects observed *in vivo*, such as improved motor coordination and regulated circadian gene expression are likely mediated through receptor-dependent signaling pathways rather than nonspecific cellular toxicity. At the same time, the persistence of circadian gene expression changes even under high-dose conditions suggests that clock gene networks are highly sensitive to disturbances in intracellular signaling and energy homeostasis. Taken together, the N2a data complement the *in vivo* results by reinforcing the strong dose dependence of caffeine's actions and by underscoring the importance of interpreting cellular findings within the physiological context established by whole-animal studies.

Caffeine can alter neuronal cell proliferation with long-term treatment, while short-term has no effect on proliferation [57]. Similarly, our *in vitro* studies on N2a cells exposed to high doses of caffeine (5 and 10 mM) revealed significant morphological changes, including rounded cell bodies and shorter dendrites. These alterations suggest that high concentrations of caffeine can disrupt neuronal cell structure, which may impact brain function and cellular health. This highlights the potential risks associated with excessive caffeine consumption and its effects on cellular morphology and viability.

## 5. Conclusions

Overall, this study offers novel and comprehensive insights into the multifaceted effects of caffeine on physiology, behavior, and the molecular regulation of circadian rhythms. The observed reduction in weight gain and enhancement of motor performance highlight caffeine's beneficial impact on both physical and cognitive functions across age groups. Importantly, the discovery that caffeine alters circadian clock gene expression and induces changes in neuronal cell morphology reveals previously underappreciated risks, particularly at higher doses. These findings emphasize the dual nature of caffeine's influence, simultaneously beneficial and potentially disruptive, underscoring its significance as a modulator of energy balance, neuroplasticity, and circadian biology. Despite these strengths, several limitations should be acknowledged. First, the behavioral assessment was restricted to a limited set of tests, which may not fully capture all the cognitive and motor effects. Second, the molecular analyses were conducted with relatively small sample sizes, reducing statistical power and increasing the possibility of type I or type II errors. Third, the study relied on a single mouse strain and two age groups, which may limit generalizability across genetic backgrounds or developmental stages. Finally, although we identified significant changes in circadian gene expression and neuronal morphology, the causal mechanisms linking these alterations to behavioral outcomes remain unresolved. Looking ahead, future work should focus on dose–response studies to define thresholds of benefit versus risk, longitudinal experiments to determine whether chronic caffeine exposure accelerates or mitigates age-related circadian decline, mechanistic investigations into adenosine receptor subtype signaling and its interplay with circadian gene networks, and translational studies in humans to evaluate whether the age-dependent effects observed in mice extend to clinical populations. Together, these directions will advance understanding of caffeine's complex interactions with biological systems and provide a foundation for safe and evidence-based consumption guidelines.

## Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

## Author Contributions

Conceptualization, YH; Data curation, SP, ZAK, AA, and UKAS; Formal analysis, SP, ZAK, AA, UKAS, AKK, YL, GL, and JK; Funding acquisition, YH and SS; Investigation, SP, ZAK, YL, GL, and JK; Methodology, YH, SP, YL, GL, SS, and JK; Project administration, YH and SS; Resources, YH and SS; Supervision, YH; Validation, YH; Visualization, SP, ZAK, UKAS, AKK, and AA; Writing-original draft, YH, ZAK, and AA; Writing - review & edit-

ing, YH, ZAK, AA, AKK, UKAS, SP, YL, GL, JK, and SS. All authors read and approved the final version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Experimental procedures using animals were approved by the Institutional Animal Care and Use Committee of Inje University (approval no. 2021-018). All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th edition, revised 2011) and relevant institutional and national guidelines for the care and use of laboratory animals. This study was conducted in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) 2.0 guidelines to ensure transparent and reproducible reporting of animal research.


## Acknowledgment

The authors sincerely thank all members of the 'Biological Clock & Aging Control' Laboratory for their valuable support and constructive comments.

## Funding

This work was supported by the Regional Innovation System & Education (RISE) Glocal University 30 program through the RISE Center, Gyeongsangnam-do, funded by the Ministry of Education (MOE) and the Gyeongsangnam-do Provincial Government, Republic of Korea (2025-RISE Glocal University 30-16-008). This study was also supported by the 'Gyeongsangnam-do Regional Innovation System & Education (RISE)' Project funded by the Ministry of Education (MOE) and Gyeongsangnam-do, Republic of Korea (RISE-202602280001).


## Conflicts of Interest

The authors declare no conflicts of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN46140.

## References


[1] Uddin MS, Sufian MA, Hossain MF, Kabir MT, Islam MT, Rahman MM, et al. Neuropsychological effects of caffeine: Is caffeine addictive. Journal of Psychology & Psychotherapy. 2017; 7: 1–12.

[2] Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacological Reviews. 1999; 51: 83–133. https://doi.org/10.1016/S0031-6997(24)01396-6

[3] Magalhães R, Picó-Pérez M, Esteves M, Vieira R, Castanho TC, Amorim L, et al. Habitual coffee drinkers display a distinct pattern of brain functional connectivity. Molecular Psychiatry. 2021; 26: 6589–6598. https://doi.org/10.1038/s41380-021-01075-4

[4] Picó-Pérez M, Magalhães R, Esteves M, Vieira R, Castanho TC, Amorim L, et al. Coffee consumption decreases the connectivity of the posterior Default Mode Network (DMN) at rest. Frontiers in Behavioral Neuroscience. 2023; 17: 1176382. https://doi.org/10.3389/fnbeh.2023.1176382

[5] Lopes CR, Cunha RA. Impact of coffee intake on human aging: Epidemiology and cellular mechanisms. Ageing Research Reviews. 2024; 102: 102581. https://doi.org/10.1016/j.arr.2024.102581

[6] Fulgoni VL, 3rd, Keast DR, Lieberman HR. Trends in intake and sources of caffeine in the diets of US adults: 2001-2010. The American Journal of Clinical Nutrition. 2015; 101: 1081–1087. https://doi.org/10.3945/ajcn.113.080077

[7] Zucconi S, Volpato C, Adinolfi F, Gandini E, Gentile E, Loi A, et al. Gathering consumption data on specific consumer groups of energy drinks. EFSA Supporting Publications. 2013; 10: 394E. https://doi.org/10.2903/sp.efsa.2013.EN-394

[8] Jaussent I, Dauvilliers Y, Ancelin ML, Dartigues JF, Tavernier B, Touchon J, et al. Insomnia symptoms in older adults: associated factors and gender differences. The American Journal of Geriatric Psychiatry: Official Journal of the American Association for Geriatric Psychiatry. 2011; 19: 88–97. https://doi.org/10.1097/JGP.0b013e3181e049b6

[9] Curless R, French JM, James OF, Wynne HA. Is caffeine a factor in subjective insomnia of elderly people? Age and Ageing. 1993; 22: 41–45. https://doi.org/10.1093/ageing/22.1.41

[10] van der Linden M, Olthof MR, Wijnhoven HAH. The Association between Caffeine Consumption from Coffee and Tea and Sleep Health in Male and Female Older Adults: A Cross-Sectional Study. Nutrients. 2023; 16: 131. https://doi.org/10.3390/nu16010131

[11] Alves ACdB, Bristot VJdO, Limana MD, Speck AE, Barros LSd, Solano AF, et al. Role of adenosine A2A receptors in the central fatigue of neurodegenerative diseases. 2019; 9: 145–156. https://doi.org/10.1089/caff.2019.0009

[12] Davis JM, Zhao Z, Stock HS, Mehl KA, Buggy J, Hand GA. Central nervous system effects of caffeine and adenosine on fatigue. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 2003; 284: R399–R404. https://doi.org/10.1152/ajpregu.00386.2002

[13] Fiani B, Zhu L, Musch BL, Briceno S, Andel R, Sadeq N, et al. The Neurophysiology of Caffeine as a Central Nervous System Stimulant and the Resultant Effects on Cognitive Function. Cureus. 2021; 13: e15032. https://doi.org/10.7759/cureus.15032

[14] Sherman H, Gutman R, Chapnik N, Meylan J, le Coutre J, Froy O. Caffeine alters circadian rhythms and expression of disease and metabolic markers. The International Journal of Biochemistry & Cell Biology. 2011; 43: 829–838. https://doi.org/10.1016/j.biocel.2011.02.008

[15] Espinosa J, Rocha A, Nunes F, Costa MS, Schein V, Kazlauckas V, et al. Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia. Journal of Alzheimer's Disease: JAD. 2013; 34: 509–518. https://doi.org/10.3233/JAD-111982

[16] Zhu Y, Hu CX, Liu X, Zhu RX, Wang BQ. Moderate coffee or tea consumption decreased the risk of cognitive disorders: an updated dose-response meta-analysis. Nutrition Reviews. 2024; 82: 738–748. https://doi.org/10.1093/nutrit/nuad089

[17] Kaster MP, Machado NJ, Silva HB, Nunes A, Ardais AP, Santana M, et al. Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered

by chronic stress. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112: 7833–7838. https://doi.org/10.1073/pnas.1423088112
[18] Grosso G, Micek A, Castellano S, Pajak A, Galvano F. Coffee, tea, caffeine and risk of depression: A systematic review and dose-response meta-analysis of observational studies. Molecular Nutrition & Food Research. 2016; 60: 223–234. https://doi.org/10.1002/mnfr.201500620
[19] Roenneberg T, Merrow M. The Circadian Clock and Human Health. Current Biology: CB. 2016; 26: R432–R443. https://doi.org/10.1016/j.cub.2016.04.011
[20] Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature. 2002; 418: 935–941. https://doi.org/10.1038/nature00965
[21] Ribeiro JA, Sebastião AM. Caffeine and adenosine. Journal of Alzheimer's Disease: JAD. 2010; 20: S3–S15. https://doi.org/10.3233/JAD-2010-1379
[22] Jagannath A, Varga N, Dallmann R, Rando G, Gosselin P, Ebrahimjee F, et al. Adenosine integrates light and sleep signalling for the regulation of circadian timing in mice. Nature Communications. 2021; 12: 2113. https://doi.org/10.1038/s41467-021-22179-z
[23] Faudone G, Arifi S, Merk D. The Medicinal Chemistry of Caffeine. Journal of Medicinal Chemistry. 2021; 64: 7156–7178. https://doi.org/10.1021/acs.jmedchem.1c00261
[24] Ripperger JA, Jud C, Albrecht U. The daily rhythm of mice. FEBS Letters. 2011; 585: 1384–1392. https://doi.org/10.1016/j.febslet.2011.02.027
[25] Potter GDM, Skene DJ, Arendt J, Cade JE, Grant PJ, Hardie LJ. Circadian Rhythm and Sleep Disruption: Causes, Metabolic Consequences, and Countermeasures. Endocrine Reviews. 2016; 37: 584–608. https://doi.org/10.1210/er.2016-1083
[26] Fredholm BB. Astra Award Lecture. Adenosine, adenosine receptors and the actions of caffeine. Pharmacology & Toxicology. 1995; 76: 93–101. https://doi.org/10.1111/j.1600-0773.1995.tb00111.x
[27] Jha PK, Bouâouda H, Gourmelen S, Dumont S, Fuchs F, Goumon Y, et al. Sleep Deprivation and Caffeine Treatment Potentiate Photic Resetting of the Master Circadian Clock in a Diurnal Rodent. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2017; 37: 4343–4358. https://doi.org/10.1523/JNEUROSCI.3241-16.2017
[28] Rees K, Allen D, Lader M. The influences of age and caffeine on psychomotor and cognitive function. Psychopharmacology. 1999; 145: 181–188. https://doi.org/10.1007/s002130051047
[29] Jee HJ, Lee SG, Bormate KJ, Jung YS. Effect of Caffeine Consumption on the Risk for Neurological and Psychiatric Disorders: Sex Differences in Human. Nutrients. 2020; 12: 3080. https://doi.org/10.3390/nu12103080
[30] Machado NJ, Ardais AP, Nunes A, Szabó EC, Silveirinha V, Silva HB, et al. Impact of Coffee Intake on Measures of Wellbeing in Mice. Nutrients. 2024; 16: 2920. https://doi.org/10.3390/nu16172920
[31] Wang Y, Lau CE. Caffeine has similar pharmacokinetics and behavioral effects via the i.p. and p.o. routes of administration. Pharmacology, Biochemistry, and Behavior. 1998; 60: 271–278. https://doi.org/10.1016/s0091-3057(97)00595-9
[32] Park JM, Kim Y, Kim H, Kim YJ. Influence of short-and long-term high-dose caffeine administration on behavior in an animal model of adolescence. Journal of Korean Biological Nursing Science. 2019; 21: 217–223. https://doi.org/10.7586/jkbns.2019.21.3.217
[33] Prediger RDS, da Silva GE, Batista LC, Bittencourt AL, Takahashi RN. Activation of adenosine A1 receptors reduces anxiety-like behavior during acute ethanol withdrawal (hangover) in mice. Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology. 2006; 31: 2210–2220. https://doi.org/10.1038/sj.npp.1301001
[34] Nakae Y, Kohro S, Hogan QH, Bosnjak ZJ. Intracellular mechanism of mitochondrial adenosine triphosphate-sensitive potassium channel activation with isoflurane. Anesthesia and Analgesia. 2003; 97: 1025–1032. https://doi.org/10.1213/01.ANE.0000077072.67502.CC
[35] Campisi P, Carmichael FJ, Crawford M, Orrego H, Khanna JM. Role of adenosine in the ethanol-induced potentiation of the effects of general anesthetics in rats. European Journal of Pharmacology. 1997; 325: 165–172. https://doi.org/10.1016/s0014-2999(97)00124-6
[36] Li JD, Hu WP, Boehmer L, Cheng MY, Lee AG, Jilek A, et al. Attenuated circadian rhythms in mice lacking the prokineticin 2 gene. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2006; 26: 11615–11623. https://doi.org/10.1523/JNEUROSCI.3679-06.2006
[37] Van der Zee EA, Havekes R, Barf RP, Hut RA, Nijholt IM, Jacobs EH, et al. Circadian time-place learning in mice depends on Cry genes. Current Biology: CB. 2008; 18: 844–848. https://doi.org/10.1016/j.cub.2008.04.077
[38] Roy K, Maji D, Deb I. Oxygen glucose deprivation impairs circadian clock genes expressions in Neuro 2A neuroblastoma cells unlike C6 glioma. Biological Rhythm Research. 2022; 53: 1191–1208. https://doi.org/10.1080/09291016.2021.1911551
[39] Su Y, Zhang Z, Li H, Ma J, Sun L, Shao S, et al. A GLP-2 Analogue Protects SH-SY5Y and Neuro-2a Cells Against Mitochondrial Damage, Autophagy Impairments and Apoptosis in a Parkinson Model. Drug Research. 2021; 71: 43–50. https://doi.org/10.1055/a-1266-3263
[40] Lu PZ, Lai CY, Chan WH. Caffeine induces cell death via activation of apoptotic signal and inactivation of survival signal in human osteoblasts. International Journal of Molecular Sciences. 2008; 9: 698–718. https://doi.org/10.3390/ijms9050698
[41] Tonkaboni A, Lotfibakhshaiesh N, Danesh P, Tajerian R, Ziaei H. Evaluation of Inhibitory Effects of Caffeine on Human Carcinoma Cells. Nutrition and Cancer. 2021; 73: 1998–2002. https://doi.org/10.1080/01635581.2020.1819344
[42] Robins MT, Lu J, van Rijn RM. Unique Behavioral and Neurochemical Effects Induced by Repeated Adolescent Consumption of Caffeine-Mixed Alcohol in C57BL/6 Mice. PloS One. 2016; 11: e0158189. https://doi.org/10.1371/journal.pone.0158189
[43] Butler TR, Smith KJ, Berry JN, Sharrett-Field LJ, Prendergast MA. Sex differences in caffeine neurotoxicity following chronic ethanol exposure and withdrawal. Alcohol and Alcoholism (Oxford, Oxfordshire). 2009; 44: 567–574. https://doi.org/10.1093/alcalc/agp050
[44] Domaszewski P. Gender Differences in the Frequency of Positive and Negative Effects after Acute Caffeine Consumption. Nutrients. 2023; 15: 1318. https://doi.org/10.3390/nu15061318
[45] Cunha RA. Different cellular sources and different roles of adenosine: A1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A2A receptor-mediated facilitation of plasticity. Neurochemistry International. 2008; 52: 65–72. https://doi.org/10.1016/j.neuint.2007.06.026
[46] Zheng G, Sayama K, Okubo T, Juneja LR, Oguni I. Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice. In Vivo (Athens, Greece). 2004; 18: 55–62.
[47] Jocken JWE, Blaak EE. Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. Physiology & Behavior. 2008; 94: 219–230. https://doi.org/10.1016/j.physbeh.2008.01.002
[48] Reverte-Salisa L, Sanyal A, Pfeifer A. Role of cAMP and cGMP Signaling in Brown Fat. Handbook of Experimental Pharmacol-

ogy. 2019; 251: 161–182. https://doi.org/10.1007/164_2018_117
[49] Morley JE. Decreased food intake with aging. The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences. 2001; 56: 81–88. https://doi.org/10.1093/gerona/56.suppl_2.81
[50] Boss GR, Seegmiller JE. Age-related physiological changes and their clinical significance. The Western Journal of Medicine. 1981; 135: 434–440.
[51] Henry CJ. Mechanisms of changes in basal metabolism during ageing. European Journal of Clinical Nutrition. 2000; 54: S77–S91. https://doi.org/10.1038/sj.ejcn.1601029
[52] Horrigan LA, Kelly JP, Connor TJ. Caffeine suppresses TNF-alpha production via activation of the cyclic AMP/protein kinase A pathway. International Immunopharmacology. 2004; 4: 1409–1417. https://doi.org/10.1016/j.intimp.2004.06.005
[53] Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. Structure and function of adenosine receptors and their genes. Naunyn-Schmiedeberg's Archives of Pharmacology. 2000; 362: 364–374. https://doi.org/10.1007/s002100000313
[54] Mahdi S, Almosawi S, Baksh H, Qareeballa A, Alsaleh B, Falamarzi F, et al. Effect of chronic administration and withdrawal of caffeine on motor function, cognitive functions, anxiety, and the social behavior of BLC57 mice. International Journal of Health Sciences. 2019; 13: 10–16.
[55] Antle MC, Steen NM, Mistlberger RE. Adenosine and caffeine modulate circadian rhythms in the Syrian hamster. Neuroreport. 2001; 12: 2901–2905. https://doi.org/10.1097/00001756-200109170-00029
[56] Golombek DA, Rosenstein RE. Physiology of circadian entrainment. Physiological Reviews. 2010; 90: 1063–1102. https://doi.org/10.1152/physrev.00009.2009
[57] Wentz CT, Magavi SSP. Caffeine alters proliferation of neuronal precursors in the adult hippocampus. Neuropharmacology. 2009; 56: 994–1000. https://doi.org/10.1016/j.neuropharm.2009.02.002