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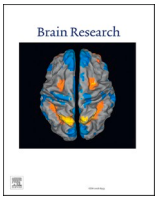

Adora2A downregulation promotes caffeine neuroprotective effect against LPS-induced neuroinflammation in the hippocampus

Paula Lemes dos Santos Sanna [a], Liebert Bernardes Carvalho [a], Camila Cristina dos Santos Afonso [a], Kassia de Carvalho [b], Rogério Aires [b], Jennyffer Souza [c], Marcel Rodrigues Ferreira [e], Alexander Birbrair [d], Maria Martha Bernardi [b], Alexandra Latini [c], Rodrigo A. Foganholi da Silva [a,b,*]

[a] Dentistry, University of Taubaté, Taubaté, São Paulo, São Paulo, Brazil
[b] Center for Epigenetic Study and Genic Regulation – CEEpiRG, Program in Environmental and Experimental Pathology, Paulista University, São Paulo, São Paulo, Brazil
[c] Laboratory of Bioenergetics and Oxidative Stress – LABOX, Department of Biochemistry, Center for Biological Sciences, Federal University of Santa Catarina, Florianopolis, Brazil
[d] Department of Dermatology, University of Wisconsin-Madison, Madison, WI, USA
[e] Molecular Genetics and Bioinformatics Laboratory, Experimental Research Unity, Botucatu Medical School, São Paulo State University, Brazil

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ABSTRACT

Caffeine has been extensively studied in the context of CNS pathologies as many researchers have shown that consuming it reduces pro-inflammatory biomarkers, potentially delaying the progression of neurodegenerative pathologies. Several lines of evidence suggest that adenosine receptors, especially A_1 and A_2A receptors, are the main targets of its neuroprotective action. We found that caffeine pretreatment 15 min before LPS administration reduced the expression of Il1b in the hippocampus and striatum. The harmful modulation of caffeine-induced inflammatory response involved the downregulation of the expression of A_2A receptors, especially in the hippocampus. Caffeine treatment alone promoted the downregulation of the adenosinergic receptor Adora2A; however, this promotion effect was reversed by LPS. Although administering caffeine increased the expression of the enzymes DNA methyltransferases 1 and 3A and decreased the expression of the demethylase enzyme Tet1, this effect was reversed by LPS in the hippocampus of mice that were administered Caffeine + LPS, relative to the basal condition; no significant differences were observed in the methylation status of the promoter regions of adenosine receptors. Finally, the bioinformatics analysis of the expanded network demonstrated the following results: the Adora2B gene connects the extended networks of the adenosine receptors Adora1 and Adora2A; the Mapk3 and Esr1 genes connect the extended Adora1 network; the Mapk4 and Arrb2 genes connect the extended Adora2A network with the extended network of the proinflammatory cytokine Il1β. These results indicated that the anti-inflammatory effects of acute caffeine administration in the hippocampus may be mediated by a complex network of interdependencies between the Adora2B and Adora2A genes.

1. Introduction

Caffeine is the most commonly consumed psychoactive drug in the world. Its consumption is related to an increase in alertness and excitement, as well as, improvement in cognitive performance (Fredholm et al., 1999a). It acts as an antagonist of two RAs, adenosine receptors (ARs) type A_1 and A_{2A}, which are the only ones other than adenosine that control the neural networks. The effects of caffeine on synaptic transmission and plasticity of the hippocampus are mediated by the selective antagonism of adenosine receptors, where A_1R is responsible for the effect of caffeine on synaptic transmission, whereas $A_{2A}R$ regulates the effects of caffeine on long-term potentiation (LTP) (Lopes et al., 2019).

Epidemiological studies have shown that caffeine consumption is inversely correlated with the risk of several neurodegenerative diseases (Ikram et al., 2020) since its habitual consumption exerts concerted

* Corresponding author at: Programa de Pós-Graduação em Ciências da Saúde, Universidade de Taubaté – UNITAU/Programa de Pós-Graduação em Patologia Ambiental e Experimental, Universidade Paulista – UNIP, Brazil.
E-mail addresses: marcel.ferreira@unesp.br (M. Rodrigues Ferreira), rodrigo.silva3@docente.unip.br (R.A. Foganholi da Silva).

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pleiotropic effects on the epigenomic, proteomic, and metabolomic levels in the hippocampus; thus, reducing processes related to metabolism while inducing specific epigenetic changes in genes related to synaptic transmission/plasticity in hippocampal neurons (Paiva et al., 2022). Therefore, researchers have long investigated the main beneficial effects of consuming coffee on human health (Socała et al., 2020) (Paiva et al., 2022).

Researchers have demonstrated the protective effects of caffeine by studying animal and prospective cohort models; some have found that its regular consumption may reduce the risk of stroke (Pham et al., 2022), coronary heart disease (Voskoboinik et al., 2019), and reduce mortality associated with cardiovascular disease (O'Keefe et al., 2018). Concerning neurodegenerative diseases, recent studies have shown that caffeine has neuroprotective effects on Parkinson's disease (Xu et al., 2002) (Sääksjärvi et al., 2008) (Yang et al., 2017) (Ren and Chen, 2020), Alzheimer's disease (Arendash and Cao, 2010), and other neurodegenerative disorders (Camandola et al., 2019). However, excessive caffeine consumption can trigger adverse effects in some people; specifically, an increase in serum total cholesterol concentration (Wei, 2002) and cardiovascular problems related to high blood pressure, tachycardia, and arrhythmia (Butt and Sultan, 2011) were reported in some studies.

The beneficial effects of caffeine consumption are predominantly due to the action on ARs (Cunha, 2016). However, although caffeine exerts its effects predominantly through non-selective antagonism of the ARs in the brain, several studies have shown that several classes of neurotransmitters, such as noradrenaline, dopamine, and acetylcholine (Ribeiro and Sebastião, 2010) (Urry and Landolt, 2015), experience significant side effects. Two of the four adenosine receptors, i.e., the A_1 receptor (A_1R) and the A_{2A} receptor ($A_{2A}R$), are highly expressed throughout the brain and are primarily responsible for the effects of adenosine (Cunha, 2005) (Hackett, 2018). Although A_1R is the most abundant and widely distributed receptor, $A_{2A}R$ is more abundant in the basal ganglia and synapses throughout the rest of the brain (Fredholm et al., 2005). Additionally, the $A_{2A}R$ receptors are expressed in astrocyte hairs and microglia; thus, they do not control Na+/K+ -ATPase and do not participate in glutamate absorption or the production of pro-inflammatory cytokines (Cunha and Agostinho, 2010) (Matos et al., 2013).

The affinity of these receptors for adenosine is different; for example, the A_1R can be activated with concentrations approaching 70 nM. In contrast, as $A_{2A}R$ has a lower affinity, it needs adenosine concentrations close to 150 nM (Dunwiddie and Masino, 2001). This difference in affinity is not observed for caffeine, and A_1R and $A_{2A}R$ receptors both have a high affinity for caffeine (Fredholm et al., 1999b). The activation of A_1R promotes the inhibition of adenylate cyclase, decreases the number of neurotransmitters released at presynaptic nerve terminals, and depresses neuronal firing at postsynaptic sites (Shen and Chen, 2009). Although the A_1R has been traditionally described as a neuroprotective receptor due to its inhibitory effects, as it decreases glutamate release and hyperpolarizes neurons (Lazarevic et al., 2021), studies have shown that prolonged A_1R activation may promote neurodegeneration (Chen et al., 2014) (Stockwell et al., 2016) (Chen et al., 2016) (Cunha, 2016). Its prolonged activation after treatment or prolonged treatment with agonists leads to the desensitization and loss of neuroprotective function; such changes can reverse its therapeutic effects and aggravate brain damage (Von Lubitz et al., 1994).

In contrast, $A_{2A}R$ activation is related to neurodegenerative effects, which leads to an excitatory modulation of the neurotransmitter controlling the release of glutamate (Lopes et al., 2002), acetylcholine (Cunha et al., 1994) and GABA (Cunha and Ribeiro, 2000) that in the hippocampus, can increase neuronal excitability and promote neuronal death (Ikram et al., 2020) (Stockwell et al., 2017). Blocking of $A_{2A}R$ greatly boosts neuroprotection in the brain preventing damage triggered by different noxious stimuli (Popoli et al., 2004) (Cunha, 2005) (Sallaberry et al., 2013).

During brain development, studies have demonstrated unbalanced alterations between the inhibitory A_1 and excitatory actions mediated by $A_{2A}R$, which vary in different brain areas. The density of $A_{2A}R$ increases as the cortex develops, but its expression in the striatum remains unaltered (Cunha et al., 1995) (Lopes et al., 1999). In contrast, empirical studies have shown that the density and immunoreactivity of the anti-A2A receptor increase in the nerve terminal of the hippocampus of aged rats (Rebola et al., 2003), as well as, in humans (Temido-Ferreira et al., 2020). This overexpression is related to memory impairment commonly observed during development (Temido-Ferreira et al., 2020).

Besides enhancing neuronal excitability, $A_{2A}R$ activation decreases A_1R functionality (Lopes et al., 2002) (Shen and Chen, 2009). A study investigated this cross-talk between A_1 and A_{2A} receptors, where the activation of $A_{2A}R$ was found to decrease the inhibition of synaptic transmission mediated by A_1R, and the superactivation of A_1R was found to block the synaptic transmission of the hippocampus (O'Kane and Stone, 1998) (Lopes et al., 2023). However, more information on the role of the adenosine receptors A_1R and $A_{2A}R$ under normal physiological conditions and neurodegenerative diseases has suggested that the molecular mechanisms underlying the anti-inflammatory action of caffeine are a promising path promissor na search for alvos, especially concerning neurodegenerative diseases (Rivera-Oliver and Díaz-Ríos, 2014). The neuroprotective action of $A_{2A}R$ related mainly to microglial reactivity and the control of neuroinflammation (Badshah et al., 2019) (Boia et al., 2016) was first demonstrated by Rebola et al. (Rebola et al., 2011).

Along with the control of inflammation, $A_{2A}R$-mediated neuroprotection is also associated with the control of synaptotoxicity (Canas et al., 2009), considering that $A_{2A}R$ plays a crucial role in synaptotoxicity-induced memory dysfunction, and caffeine consumption prevents synaptic dysfunction and astrogliosis (Cognato et al., 2010) (Duarte et al., 2012). The direct effects that the overexpression of $A_{2A}R$ in hippocampal neurons has on the activation of NMDA receptors with consequent synaptic dysfunction and memory impairment cannot be excluded (Rebola et al., 2008) (Temido-Ferreira et al., 2020), like too synaptotoxicity often associated with neurodegeneration events independent of neuroinflammation (Canas et al., 2018).

As caffeine consumption has beneficial effects on the aging process in healthy individuals and those with neurodegenerative dementia, researchers speculate that it may be implemented as a potential therapeutic strategy. In this study, we investigated the molecular mechanisms underlying the neuroprotective effect of caffeine, especially the involvement of DNA methylation in the transcriptional control of adenosine receptors, as studies have shown that certain components in coffee can modulate the activity of enzymes involved in the transfer of the methyl group in the DNA molecule (Lee and Zhu, 2006) and alter gene expression (Chuang et al., 2017). Based on the underlying assumption that neuroinflammation is directly associated with damage to neurons during the aging process, we investigated the molecular mechanisms underlying the neuroprotective effect of caffeine.

2. Results

2.1. Caffeine pretreatment prevents LPS-induced neuroinflammation and alters the expression of A1R and A2AR receptors

The effects of caffeine on the expression of *Il 1β, A_1R,* and *$A_{2A}R$* were evaluated in the striatum and hippocampus of mice that were administered caffeine (6 mg/kg) 15 min before LPS administration (0.33 mg/kg). The results showed that the pro-inflammatory gene *Il 1β* was positively regulated in the striatum (Fig. 1a) and hippocampus (Fig. 1d). Additionally, the increase in the expression of the pro-inflammatory gene *Il 1β* induced by LPS was hampered by caffeine. The interaction between caffeine and LPS was significant in the striatum [$F(1,16) = 7.29$; $P < 0.01$] and the hippocampus [$F(1,16) = 24.11$; $P < 0.001$] (Fig. 1a and d), after the increased level of *Il 1β* was normalized. Furthermore, positive modulation of the expression of the *A1R* receptor

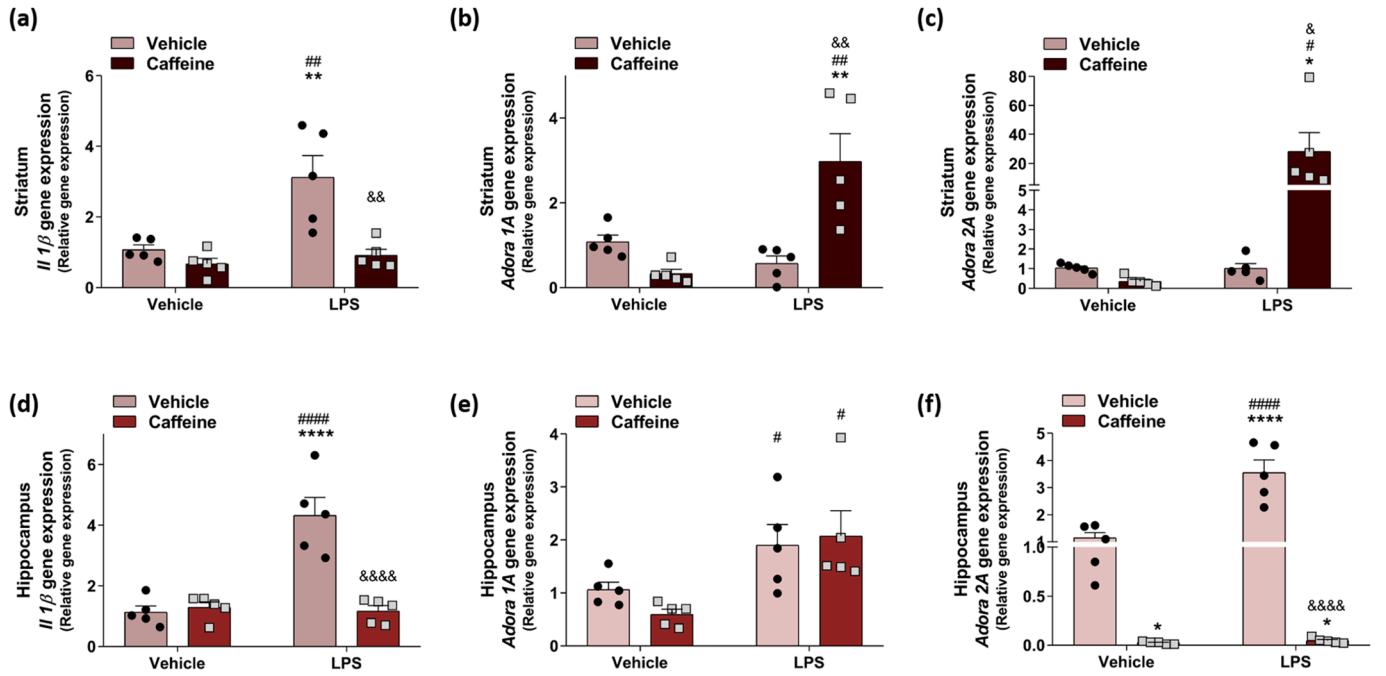

Fig. 1. Gene expression of the adenosine receptors Adora1 (A_1R) and Adora2A ($A_{2A}R$) in the brain of mice that were administered lipopolysaccharide (LPS). Total RNA was extracted using the TRIzol®/chloroform/isopropanol method. The transcription profile of the *Il 1β*, *Adora1*, and *Adora2A* genes in the striatum (a, b, and c) and hippocampus (d, e, and f) was evaluated by qPCR. The gene expression data were normalized by the average of the Ct of the *18 s*, *Gapdh*, and *β-actin* genes and calculated by the $2^{-\Delta Ct}$ method. The bars represent the mean ± standard deviation of five independent animals used in technical replicates; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for vehicle versus Caffeine; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for vehicle versus LPS; & P < 0.05, && P < 0.01, and &&& P < 0.001 for vehicle versus Caffeine + LPS.

[F (1,16) = 19.99; P < 0.001] and *$A_{2A}R$* [F (1,16) = 4.38; P = 0.05] was found only in the striatum of mice in the Caffeine-group, with no significant effects observed for the separate administration of caffeine and LPS (Fig. 1b and c). Moreover, in the hippocampus of animals treated with LPS, the expression of *$A_{2A}R$* was substantially upregulated, but this increase was completely reversed by caffeine (Fig. 1e and f). On the other hand, in the Caffeine-group, *$A_{2A}R$* expression was significantly and negatively modulated [$F_{(1,16)} = 21.99$; $P < 0.001$] (Fig. 1f), but the effect induced by LPS on *A_1R* transcript levels (Fig. 1e) was not modified.

2.2. LPS and/or caffeine modulated the expression of genes encoding DNA-modifying enzymes

To evaluate whether exposure to LPS and/or caffeine alters the expression of the genes encoding enzymes related to DNA methylation, we initially assessed the expression of genes that encode DNA-modifying enzymes. The results obtained for the expression of Dnmts are shown in Fig. 2a – f. The group treated only with caffeine showed significantly higher expression of *Dnmt1* (responsible for maintaining DNA methylation patterns) [F(1,15) = 9.17; P < 0.01] (Fig. 2d) and *Dnmt3b* (Dnmt3a and b are responsible for de novo DNA methylation) [F (1,16) = 49.27; P < 0.001] only in the hippocampus (Fig. 2d and f). However, *Dnmt3a* expression was significantly reduced in the striatum [F (1,16) = 11.85; P < 0.01] (Fig. 2b). LPS administration induced effects opposite to those observed with caffeine. LPS significantly decreased the expression of *Dnmt1* [F (1,15) = 65.60; $P < 0.001$] (Fig. 2d) and increased the expression of *Dnmt3a* in the hippocampus (Fig. 2e). For the expression of the *Dnmt1* and *Dnmt3b* genes, no significant effects of caffeine and LPS were recorded in the striatum (Fig. 2a and c) and for the expression of *Dnmt3a* in the hippocampus after treatment only with caffeine (Fig. 2e). Additionally, in the group in which the animals were pretreated with caffeine and then administered LPS, the expression of *Dnmt1* and *Dnmt3b* in the striatum was positively modulated (Fig. 2a and c) and the expression of *Dnmt1* in the hippocampus decreased (Fig. 2d).

Furthermore, caffeine in the striatum did not alter the level of expression of the Tet methylcytosine dioxygenase (TET) members evaluated (Fig. 2g – i), whereas, LPS significantly upregulated the expression of *Tet1* [F (1.16) = 5.645; $P < 0.03$] (Fig. 2g). In contrast, in the hippocampus, treatment with caffeine and LPS together [F (1,16) = 46.811; P < 0.000003] and LPS alone [F (1,16) = 26.331; P < 0.0001] significantly downregulated *Tet1* expression (Fig. 2j). A significant positive interaction was found between caffeine and LPS pretreatment for the upregulation of the expression of *Tet2* [F (1.14) = 5.065; $P < 0.04$] (Fig. 2h) and *Tet3* [F (1.16) = 21.138; $P < 0.0003$] (Fig. 2i); however, caffeine reversed the increase in gene expression of the enzyme *Tet1* promoted by the treatment of LPS in the striatum [F (1.16) = 5.645; $P < 0.03$] (Fig. 2g). In the hippocampus, a positive interaction was found only for the upregulation of the expression of the *Tet2* gene [F (1.16) = 37.455; $P < 0.00001$] (Fig. 2k).

2.3. Caffeine and LPS treatment alter the epigenetic landscape of the Adora2A gene in the SNC

Based on the above results, which showed alterations in the expression of DNA-modifying enzymes, we evaluated whether the adenosine receptors A_1R and $A_{2A}R$ were epigenetically regulated. First, we found that the treatments administered altered $A_{2A}R$ epigenetically to the greatest extent among the genes investigated. Regarding the percentage of methylation (5-meC) and hydroxymethylation (5-hmeC) marks, the results of the two-way ANOVA revealed a significant effect of caffeine on the 5-meC mark [F (1.16) = 19.664; $P < 0.0004$], LPS [F (1.16) = 5.640; $P < 0.03$], and a significant interaction between caffeine and LPS [F (1.16) = 84.900; $P < 0.001$] (Fig. 3a). Caffeine significantly decreased the percentage of the 5-hmeC mark [F (1.16) = 3.280; $P < 0.005$] (Fig. 3d), but the other treatments did not have significant effects. In contrast, in the hippocampus, the rate of 5-meC [F (1.16) = 16.008; $P < 0.001$] and 5-hmeC [F (1.16) = 17.910; $P < 0.0006$] marks decreased significantly only in the LPS group (Fig. 4a and d).

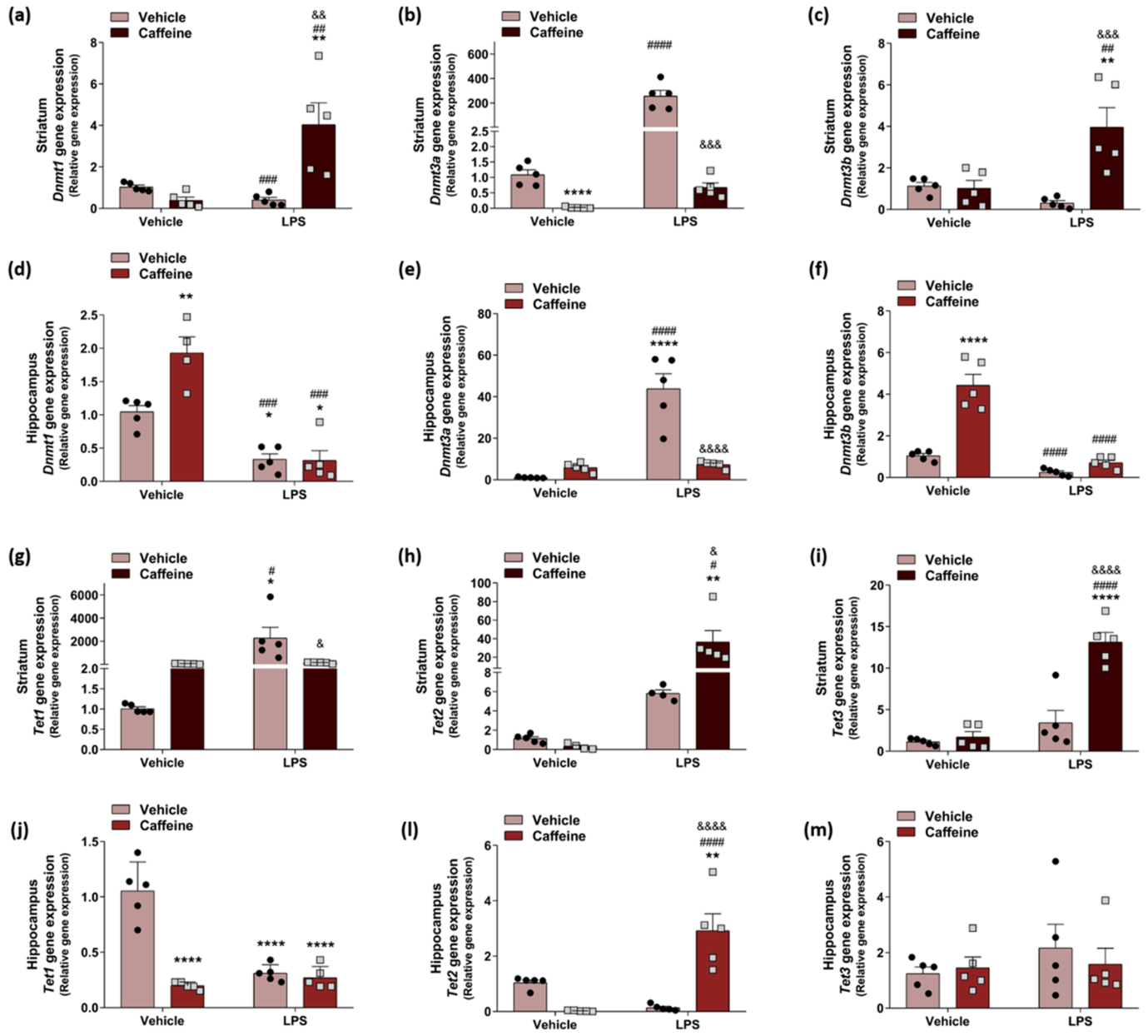

Fig. 2. Effects of caffeine and LPS treatment on the gene expression of DNA-modifying enzymes. Analysis of the gene expression of *Adora1* and *Adora2A* genes in brain structures. After total RNA was extracted using the TRIzol®/Chloroform/Isopropanol method, the transcriptional profiles of the Dnmts [*Dnmt1* (a, d), *Dnmt3a* (b, e), and *Dnmt3b* (c, f)] and Tets [*Tet1* (g, j), *Tet2* (h, k), and *Tet3* (i, l)] genes in the striatum and hippocampus, respectively, were evaluated by qPCR in the brain. The gene expression results were normalized by the average of the Ct of the *18 s*, *Gapdh*, and *β-actin* genes and calculated by the $2^{-\Delta Ct}$ method. Bars represent the mean ± standard deviation of five independent animals used in technical replicates; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for vehicle versus Caffeine; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for vehicle versus LPS; & $P < 0.05$, && $P < 0.01$, and &&& $P < 0.001$ for vehicle versus Caffeine + LPS.

Two CpG islands were identified in the gene encoding the adenosine 1 receptor, *Adora1*. The results obtained for the CpG 1 island, located at chr1:134234705–134235089, revealed a significant effect only for the interaction between the treatments; specifically, the interaction significantly decreased the percentage of the 5-hmeC mark in the striatum (Fig. 3e). A significant effect of caffeine ($P < 0.02$) and LPS ($P < 0.03$) was revealed by multiple comparison analyses, and their interaction also had a significant effect [$F(1.16) = 15.611$; $P < 0.001$] (Fig. 4e). No significant differences were recorded for the 5-meC mark (Fig. 3b and b). Additionally, no significant differences in the percentage of the evaluated marks were observed in the CpG 2 island (chr1:134235378–134235655) in the striatum (Fig. 3c and f) and the hippocampus (Fig. 4c and f).

2.4. Epigenetic reprogramming of adenosine receptors by caffeine and LPS did not directly mediate A_1R and $A_{2A}R$ gene expression

To further assess the involvement of the changes in the epigenetic landscape presented above, we determined the 5-meC/5-hmeC ratio and performed a correlation analysis between the epigenetic changes and their effective expression of the corresponding genes. The ratio of 5-hmeC/5-meC reflects the total methylation status of the gene, with higher ratios indicating demethylation and active transcription (Mellen et al., 2012). In this context, we detected a significant increase in the 5-meC/5-hmeC ratio only in the hippocampus at the CpG 2 island of the *Adora1* (A_1R) gene after LPS treatment, as determined by the multiple comparison analysis ($P < 0.02$) (Fig. 4i). No other significant differences were observed for the 5-meC/5-hmeC ratio (Fig. 3g – i) and (Fig. 4g and

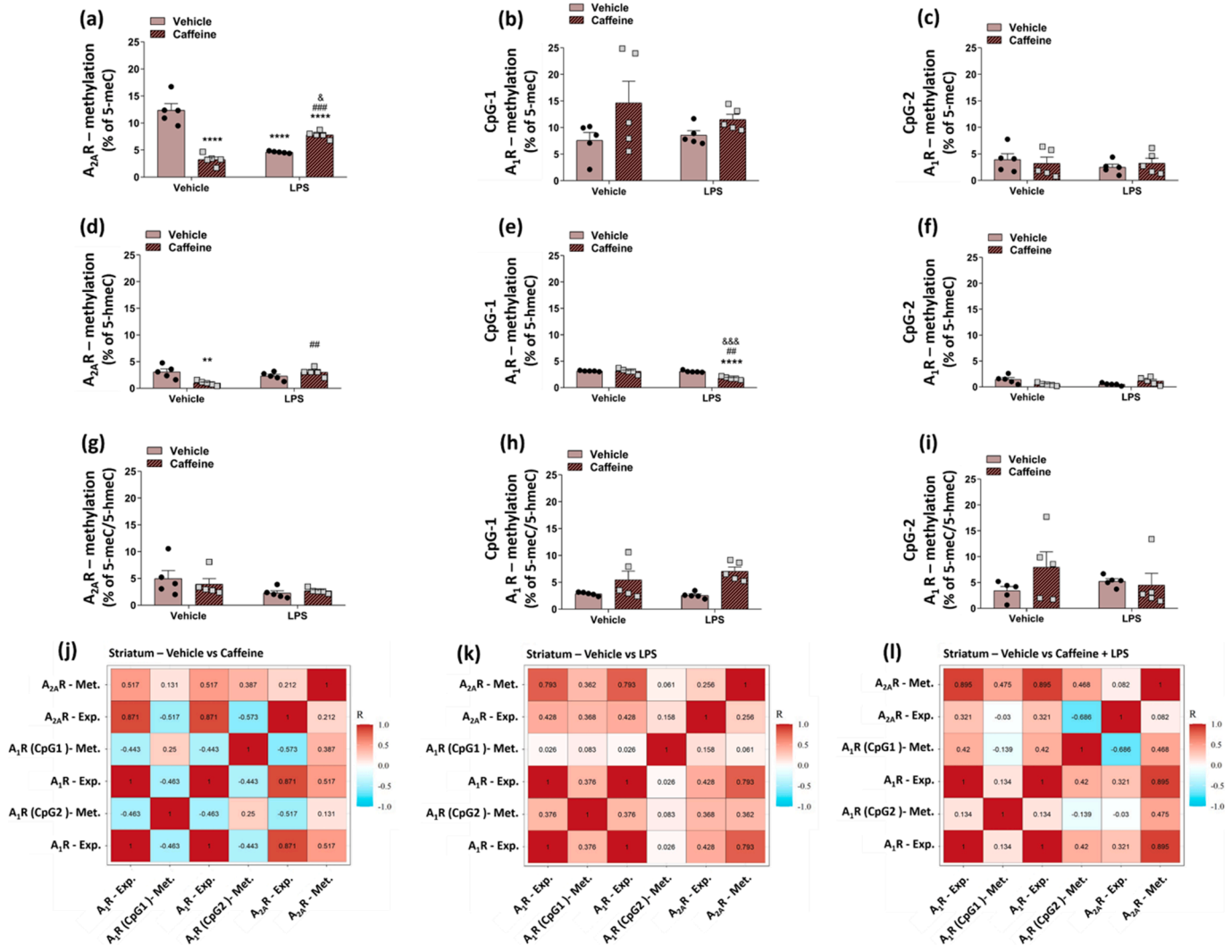

Fig. 3. Effect of caffeine and LPS treatment on promoter methylation of the Adora1 (A_1R) and Adora2A ($A_{2A}R$) genes in the striatum. After total DNA was extracted using the phenol/chloroform protocol method, the genomic DNA was treated with T4-β-glucosyltransferase (T4-BGT), followed by digestion with endonucleases. The percentage of the 5-meC and 5-hmeC marks of the genes of $A_{2A}R$ (a, d, g) and A_1R [CpG1 (b, e, h) and CpG2 (c, f, i)] were determined by the cycle threshold (Ct) method. The methylation results are presented as the *Hpa*II levels – *Msp*I levels/control levels and the hydroxymethylation results are presented as the *Msp*I levels-control levels. Correlation analysis was performed between gene promoter DNA methylation/hydroxymethylation pattern and gene expression of $A_{2A}R$ and A_1R for the groups vehicle vs. Caffeine (j), vehicle vs. LPS (k), and vehicle vs. Caffeine + LPS (l). Bars represent the mean ± standard deviation of five independent animals used in technical replicates; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for vehicle versus Caffeine; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for vehicle versus LPS; & $P < 0.05$, && $P < 0.01$, and &&& $P < 0.001$ for vehicle versus Caffeine + LPS; positive correlation between $r = 0.6$ and 1.

h).

We further analyzed the relationship between the expression of the A_1R and $A_{2A}R$ genes and their promoter methylation status, where we tested the correlation between the epigenetic changes and the effective expression of the corresponding genes by conducting Pearson correlation analyses. The results for the striatum (Fig. 3j – l) and the hippocampus (Fig. 4j – l) showed no correlations with product moment above $r = 0.6$, and the methylation patterns of the promoter regions were not inversely proportional to the effective expression. The correlations between the expression of the A_1R ($r = 0.793$) and $A_{2A}R$ ($r = 1.0$) genes were significant only in the group treated with caffeine in the striatum and the hippocampus (Fig. 3j and j).

Next, to better understand the relationship between the expression of the Adora1 and Adora2A genes and their association with the inflammatory response, we used the genes *Adora1*, *Adora2A,* and *Il 1β* as input for bioinformatics analysis. Because the STRING database did not have information on the direct interactions between these three proteins, we built expanded networks for each one. The Adora1 expand network included 114 proteins with 1,730 interactions (Fig. 5a), Adora2A included 118 proteins with 1,654 interactions (Fig. 5b), and Il 1β included 121 proteins with 2,570 interactions (Fig. 5c). The Adora1 and Adora2A networks have 40 shared proteins, whereas, both share only two proteins each with the Il 1β network. No protein common to the three networks was detected (Fig. 5d). Then, the networks were merged into a single network containing 309 proteins and 5,594 interactions (Fig. 6).

To better understand the relationship between the three networks, all simple paths were calculated starting from *Adora1* or *Adora2A* and ending at *Il 1β*. To save computational time, the cutoff criteria were set for paths of up to six proteins and were represented in yellow for proteins related only to the pathway activated by Adora1, in orange for proteins that were part of the pathway mediated by Adora2A, and in red for the proteins common between the two adenosine receptors. (Fig. 6a). In the black frame, we highlighted the interactions between the proteins Nrlp3 (68 interactions), Asc1 (32 interactions), and Caspase 1 (84 interactions), which are the components of the inflammasome complex

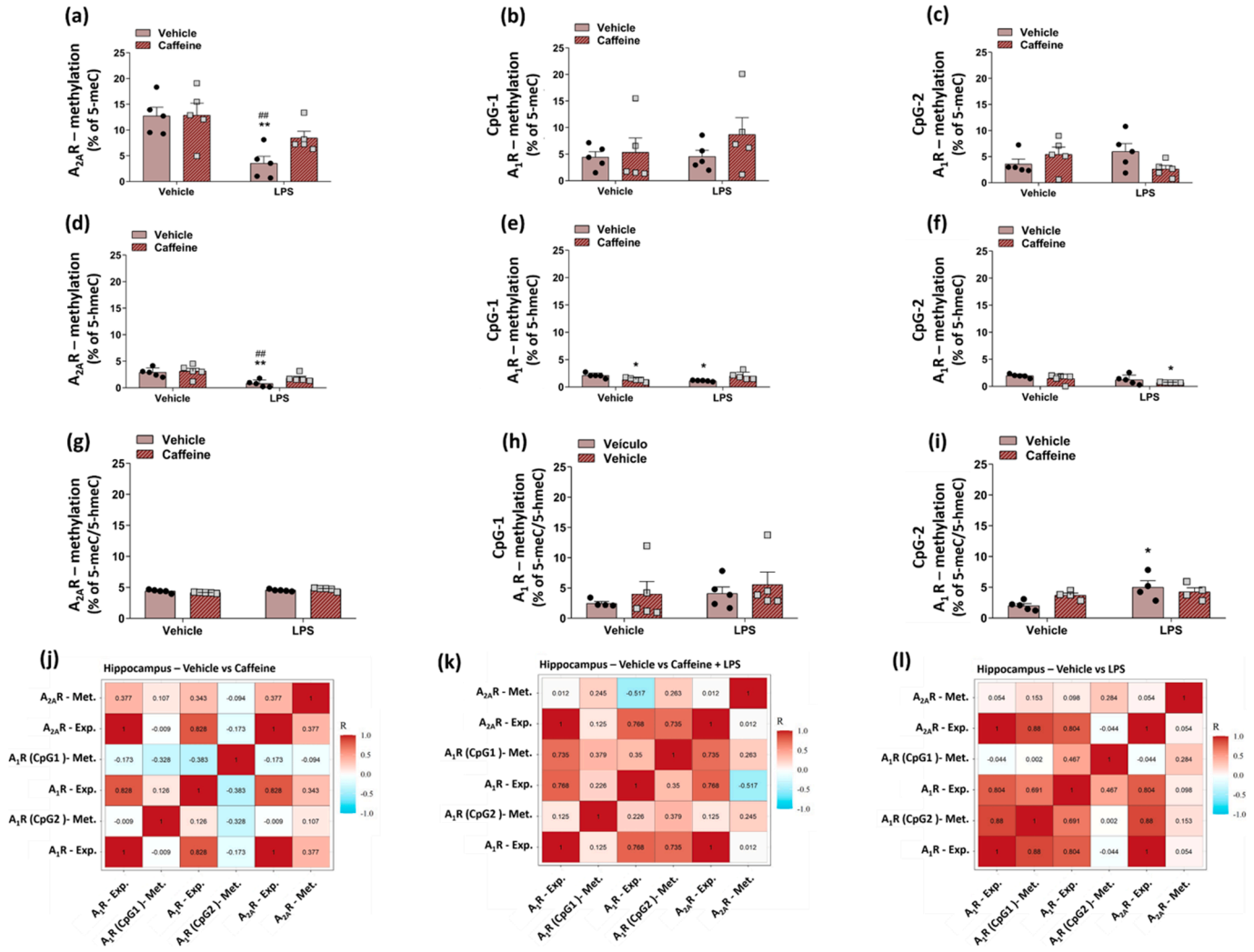

Fig. 4. The effect of caffeine and LPS treatment on promoter methylation of the Adora1 (A_1R) and Adora2A ($A_{2A}R$) genes in the hippocampus. After total DNA was extracted by the phenol/chloroform protocol method, the genomic DNA was treated with T4-β-glucosyltransferase (T4-BGT) followed by digestion with endonucleases. The percentage of the 5-meC and 5-hmeC marks of the $A_{2A}R$ (a, d, g) and A_1R genes [CpG1 (b, e, h) and CpG2 (c, f, i)] were determined by the cycle threshold (Ct) method. The results of methylation are presented as *Hpa*II levels – *Msp*I levels/control levels and the hydroxymethylation results are presented as *Msp*I levels-control levels. The correlation analysis between gene promoter DNA methylation/hydroxymethylation pattern and gene expression of $A_{2A}R$ and A_1R for the groups Vehicle vs. Caffeine (j), Vehicle vs. LPS (k), and Vehicle vs. Caffeine + LPS (l). Bars represent the mean ± standard deviation of five independent animals used in technical replicates; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for vehicle versus caffeine; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for vehicle versus LPS; & $P < 0.05$, && $P < 0.01$, and &&& $P < 0.001$ for vehicle versus Caffeine + LPS; positive correlations were significant at r 0.683 ≤ r ≤ 1. positive correlation between r = 0.6 and 1.

and responsible for activating Il 1β (Fig. 6b). The large number of proteins shared between the Adora1 and Adora2a networks (represented in red) indicated a high correlation between the biological processes triggered by the receptors. However, we highlighted in the red frame four proteins that were highly related, including Esr1 (116 interactions) and Mapk3 (176 interactions), which were common only to the Adora1/Il 1β network, and Arrb2 (108 interactions) and Mapk14 (206 interactions) common only to the Adora2A/Il 1β network (Fig. 6c), as shown in the Venn diagram (Fig. 5d). We also highlighted the Adora2B (24 interactions) receiver as a critical point in the connection between the Adora1 and Adora2A networks, represented in the violet frame (Fig. 6d).

The proteins that were a part of different pathways were used to conduct an enrichment analysis of the KEGG pathways and Gene Ontologies (Kanehisa et al., 2017). The results were filtered for pathways and ontologies with *Il1β*. After filtering the eight most significant ontologies related to neural processes, we found that Adora1 and Adora2 had the same ontologies, and only their p-values were different; the processes were related to neuronal death (Tables S1 and S2). The simple paths starting from Adora1 and Adora2a shared all 38 KEGG pathways (Fig. 6e). The biological process enriched by the set of simple paths had 338 ontologies in common, and eight ontologies were exclusive to the paths of the samples from Adora1. In contrast, paths from Adora2A did not present exclusive ontologies (Fig. 6f). Finally, the molecular functions showed only five ontologies, and all functions were common to the set of paths from Adora1 and Adora2A (Fig. 6g).

Based on the bioinformatics analysis of the expanded network, the Adora2B gene was found to connect the extended networks of the *Adora1* and *Adora2A* genes. Gene expression was evaluated to confirm the involvement of the genes detected as central hubs of connections. The results showed that caffeine and LPS decreased the expression of the *Adora2B* gene in the hippocampus. This effect was potentiated in the Caffeine + LPS group (Fig. 6h). In the striatum, treatment with caffeine did not change the expression of the *Adora2B* gene. LPS increased (Fig. 6i) in both structures, and the effect was potentiated in the Caffeine + LPS group. Additionally, LPS treatment increased the expression of the *Ampk14* gene and decreased the expression of the

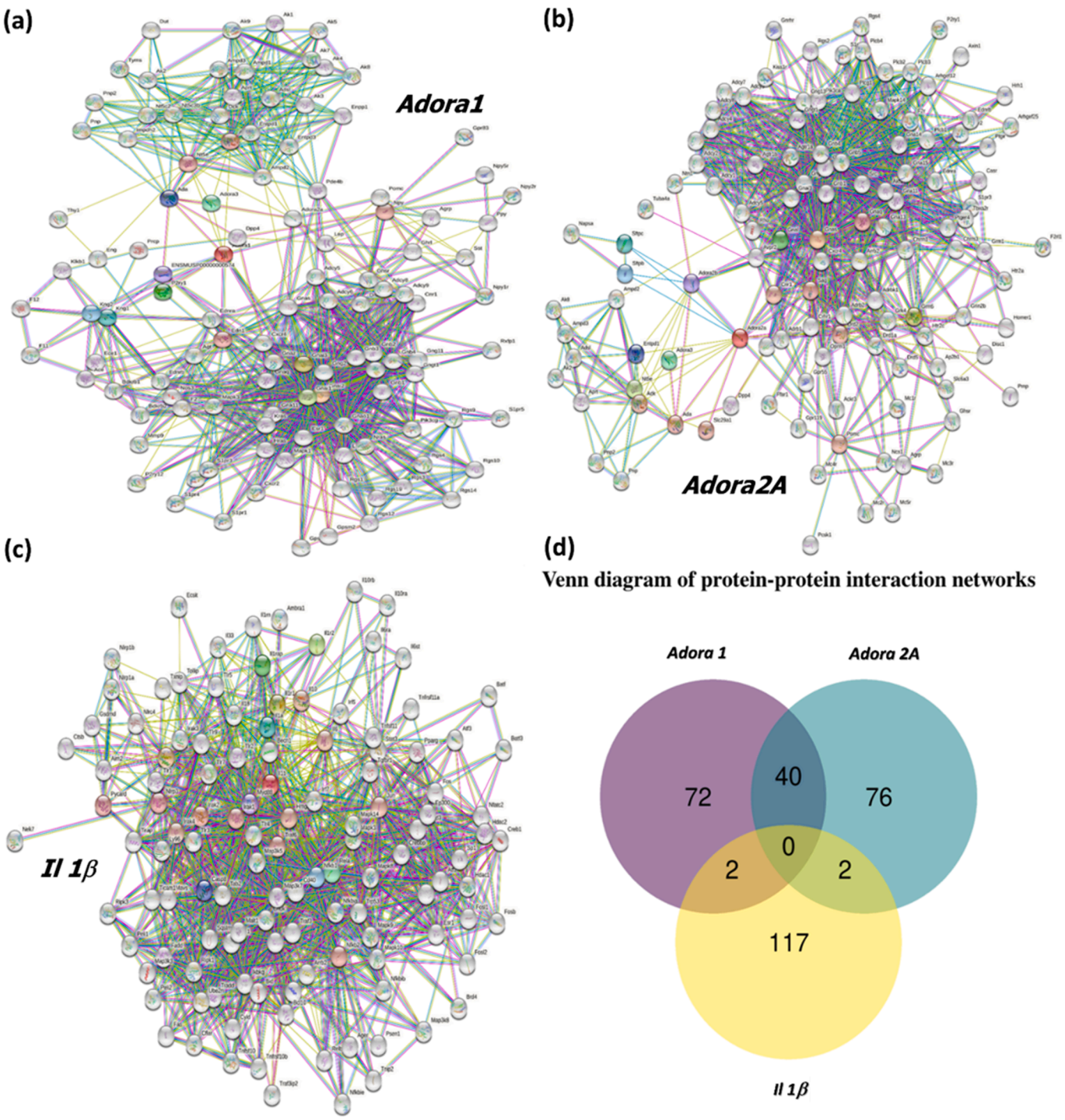

Fig. 5. Extended network. (a) *Adora1* expand network, (b) *Adora2A* expand network, and (c) *Il 1β* expand network.

Ampk3 gene in the hippocampus, and this effect was reversed by pretreatment with caffeine (Fig. 6j and n). In contrast, in the striatum, a significant increase in the expression of both genes (*Ampk13* and *Ampk14*) was observed only in the group LPS (Fig. 6k and o). A substantial increase in the expression of the *Arrb2* gene was observed only in the striatum in the LPS and Caffeine + LPS groups (Fig. 6l and m). The expression of the Esr1 gene in the hippocampus decreased in the LPS and Caffeine + LPS groups (Fig. 6p) and increased in the Caffeine + LPS group in the striatum (Fig. 6q).

However, the neuroprotective effect mediated by A2AR in controlling synaptotoxicity could not be excluded (Canas et al., 2009). Moreover, the deleterious effect on synaptic dysfunction and memory impairment due to the activation of NMDA receptors by overexpression of $A_{2A}R$ also could not be excluded (Rebola et al., 2008).

3. Discussion

Caffeine is the most consumed psychostimulant in the world, and epidemiological studies have shown that consuming caffeine can reduce the risk of several neurological and neurodegenerative diseases (Kempuraj et al., 2016) (Higdon and Frei, 2006) (Ascherio and Schwarzschild, 2016). Moreover, its anti-inflammatory action in the central nervous

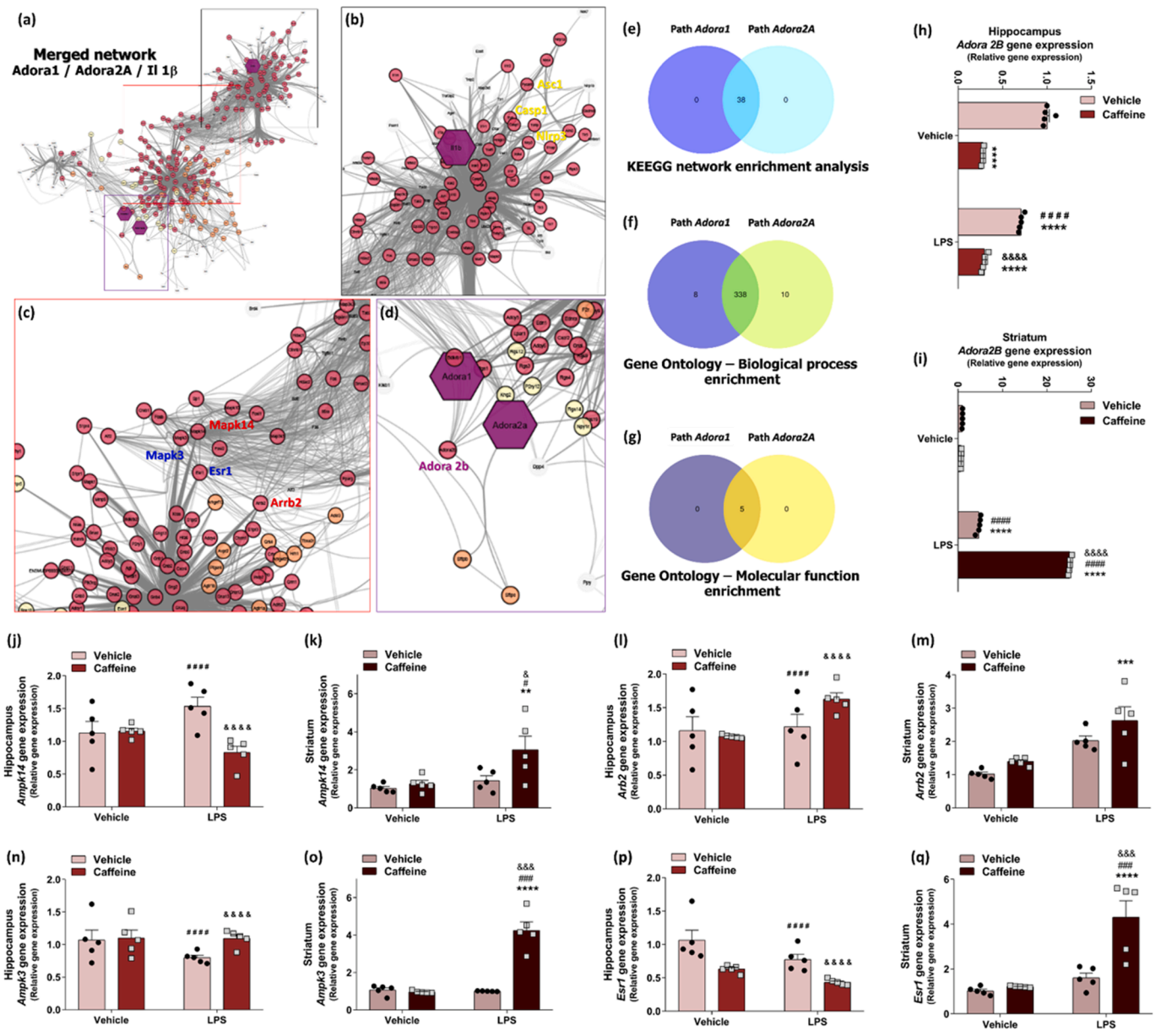

Fig. 6. Extended network for Adora1/Adora2A/Il 1β merge and validation of gene expression. (a) The merged network highlighted using purple hexagons includes the three input proteins (Adora1, Adora2A, and Il 1β). (e) A Venn diagram of the KEGG pathways in the simple path from Adora1 or Adora2a to Il1b. (f) A Venn diagram of biological process ontologies in the simple path from Adora1 or Adora2A to Il 1β. (g) A Venn diagram of molecular function ontologies in the simple path from Adora1 or Adora2A to Il 1β. Analysis of the expression of the *Adora2B* (h, i), *Ampk14* (j, k), *Arrb2* (l. m), *Amplk3* (n, o), and *Esr1* (p, q) genes in brain structures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

system was demonstrated for the first time by Rebola et al. (Rebola et al., 2011) and other groups of researchers (Brothers et al., 2010) (Badshah et al., 2019). Our results provide greater insights into the neuroprotective effect of acute pre-treatment with caffeine on a classic model of neuroinflammation induced by i.p. injection of LPS. Additionally, through bioinformatics analysis, we created an expanded network and identified new potential therapeutic targets, for the neuroprotective action of caffeine.

The dose of caffeine administered was 6 mg/kg, which was based on data from other studies in which it was found that this dose is moderate for humans, i.e., this dose has ergogenic effects without adverse effects (Connell et al., 2016) (Stear et al., 2010) (Guest et al., 2021). In the body, physiological levels of caffeine target only A_1R and $A_{2A}R$, where A_1R contributes to the effect of caffeine on synaptic transmission, and $A_{2A}R$ regulates the effect of caffeine on LTP (Lopes et al., 2019). While conducting experiments, we used the same concentration of caffeine, as the results obtained would help better elucidate the molecular mechanisms involved in the neuroprotective effect of caffeine and could be applied to humans.

Several review articles by various research groups have discussed the neuroprotective effects of caffeine on humans and animals recorded after repeated exposure (Cunha, 2016) (Arendash et al., 2009) (Kolahdouzan and Hamadeh, 2017). However, a study recently published by our research group that showed the anti-inflammatory effect of acute caffeine pre-treatment (6 mg/kg) on the expression of genes linked to epigenetic and oxidative metabolism in the vastus lateralis muscle of mice subjected to inflammation induced by lipopolysaccharide (LPS) was decisive of our choice from this experimental model (Eichwald et al., 2023).

The high levels of transcripts of the gene encoding the pro-

inflammatory cytokine Il 1β, observed in the striatum and hippocampus in the LPS group, validated our model of neuroinflammation and matched the findings of other studies previously published by our group, in which LPS was administered intraperitoneally (de Paula Martins et al., 2018) and demonstrated glial activation, characterized by an increase in immunohistochemical staining for Gfap for the different hippocampal regions (CA1, CA2, and CA3) (Carvalho et al., 2023). Other studies have found that LPS administration can activate several signaling pathways related to cell death, which, in turn, can trigger neurodegeneration and memory deficits (Khan et al., 2019b) (Batista et al., 2019). As only a few studies have investigated the characterization of the molecular mechanisms involved in the anti-inflammatory and neuroprotective effect of acute pretreatment with caffeine, we conducted this study using a model of neuroinflammation associated (or not) with caffeine (15 min before). Although in humans, the peak concentration of caffeine occurs after 30 min of administration, in mice, the peak caffeine concentration is recorded after 15 min of administration, with an elimination half-life ranging from 31.6 min to 33.5 min in both active and resting animals (article under publication). The anti-inflammatory effect of caffeine on muscles under lipopolysaccharide-induced inflammation was described by our research group (Eichwald et al., 2023).

Pro-inflammatory cytokines, especially Il 1β, play a role in activating the inflammatory response in the CNS, and neuroinflammation plays a critical and central role during the development of neurodegenerative diseases (Kempuraj et al., 2016) (Schain and Kreisl, 2017). The results presented in this study showed for the first time that the i.p. injection of caffeine 15 min before LPS could neutralize the pro-inflammatory effect of LPS, reducing the level of expression of *Il 1β* to baseline levels. In another study, it was found to be related to the protective potential of caffeine, such as the reduction of oxidative stress through regulation of the levels of Nrf2 and HO-1 in a murine model (Khan et al., 2019a). Moreover, the intracerebrovascular co-administration of LPS and caffeine was found to decrease the content of Toll-like receptor 4 (TLR4), phosphonuclear factor kappa B (p-NF-kB), and n-terminal phospho-c-Jun kinase (p-JNK) (Badshah et al., 2019). However, the most studied and well-known mechanism is associated with the antagonistic effect on adenosine receptors (Volkow et al., 2015).

Regarding adenosine receptors, A1Rs prevent the onset of damage, however, their action can be desensitized after prolonged activation. On the other hand, blocking $A_{2A}R$ was shown to facilitate neuroprotection in different models of neurodegenerative diseases (Cunha, 2016). Specifically, blocking $A_{2A}R$ imparts robust neuroprotection in different brain conditions and its chemical blockade prevents LPS-induced phosphorylation of c-jun N-terminal kinase and p38, as well as, the activation of caspase 3 (Rebola et al., 2011); moreover, $A_{2A}R$ blockade also reverses microglial inflammation (Gyoneva et al., 2014). The results presented in this study showed that acute caffeine treatment associated with LPS significantly decreased the expression of $A_{2A}R$ in the hippocampus. As the mechanism underlying the cell-specific $A_{2A}R$ receptor function in the control of neurodegeneration remains undescribed, our conclusions are limited. Therefore, researchers need to identify compounds that can simultaneously reinforce A_1R preconditioning and block the overactivation of $A_{2A}R$ to achieve greater neuroprotective effects (Cunha, 2016).

Most studied and known mechanisms are associated with the antagonistic effect on adenosine receptors (Volkow et al., 2015). It was reported that caffeine-mediated neuroprotection by adenosine $A_{2A}R$ over function (Cunha, 2016) is associated with synaptotoxicity (Canas et al., 2009) (Cognato et al., 2010) (Duarte et al., 2012) due to its direct effects on the control of NMDA receptors (Rebola et al., 2008) (Temido-Ferreira et al., 2020), which is the primary mechanism underlying the synaptotoxicity and neurodegeneration independent of neuroinflammation (Canas et al., 2018). However, our findings do not provide sufficient evidence to exclude the role of the $A_{2A}R$-mediated control of neuroinflammation.

Most biological effects of caffeine are related to its non-selective competitive antagonist action on ARs, which can modulate brain functions, such as sleep, cognition, learning, and memory (Butt and Sultan, 2011) (Canas et al., 2009). However, the mechanisms underlying its beneficial effects on the CNS are not fully elucidated. In this study, the two areas of the brain investigated responded very differently. In the striatum, we observed a significant modulation of the expression of ARs only through interactions between treatments. The isolated treatments (LPS or caffeine) did not change the expression of the ARs. In contrast, in the hippocampus, caffeine, and LPS showed opposite results; while caffeine repressed the transcription of the Adora2A receptor gene, LPS increased its expression. The results observed in the hippocampus were similar to the findings reported in other studies in which a pro-inflammatory effect was found to be associated with $A_{2A}R$ (Fu et al., 2019), and caffeine was found to negatively modulate the expression of the *Adora2A* gene (Cunha and Agostinho, 2010) (Nehlig, 2010). We hypothesized that this difference observed between the structures can be explained, at least partly, by the differential distribution of these ARs in the CNS. Among the different ARs, A_1R and $A_{2A}R$ have a high affinity for caffeine. However, A_1R is more abundant in the CNS, with high density in the cerebral cortex, hippocampus, cerebellum, thalamus, brainstem, and spinal cord (Fredholm et al., 2011) (Fredholm et al., 1999a). The expression of $A_{2A}R$ is more restricted, occurring preferentially in the striatum, nucleus accumbens, and olfactory tubercle (Fredholm et al., 1999b). We cannot rule out that adenosine-mediated signaling via A_1 and A_{2A} receptors are distinct, considering that $A_{2A}R$ activation elicits an antagonistic response to those observed for A_1R (Liu et al., 2019) (Kessey and Mogul, 1998).

Regarding the anti-inflammatory action of caffeine, caffeine inhibits the activation of the Nlrp3 inflammasome by suppressing the MAPK/NF-κB signaling pathway and the production of ROS associated with $A_{2A}R$ in macrophages. It also decreases the expression of caspase 1, which activates the maturation of the pro-inflammatory cytokines IL-1β and IL-18 (Zhao et al., 2019) (Chen et al., 2020) (Guo et al., 2015). Based on this finding, we hypothesized that the anti-inflammatory action of caffeine, specifically in the hippocampus, is related to the $A_{2A}R$/Nrlp3 inflammasome axis since we found a reduction in the expression of $A_{2A}R$.

Concerning transcriptional control, the number of published studies on the involvement of epigenetic mechanisms, especially related to the functions of the CNS in adults (Sweatt, 2013) (Cholewa-Waclaw et al., 2016), increased considerably in the last decade. Specifically, studies on neuronal physiology have shown significant associations between epigenetic changes and the development of neurological disorders (Christopher et al., 2017). In this context, the characterization of the effect of LPS and caffeine pretreatment on the transcriptional control of DNA-modifying enzymes is important. Transcriptional characterization of genes that encode DNA-modifying enzymes in this study revealed that brain structures respond differently to treatments for most enzymes evaluated. The findings of previous studies have indicated that the increase in Dnmts enzymes is mainly related to their de novo methylation capacity, which is necessary for the rapid transcriptional regulation of neuronal genes during neuronal maturation and synaptic plasticity (Feng et al., 2005) (Symmank and Zimmer, 2017) (Bayraktar and Kreutz, 2018).

The TET enzymes might play a role in the dynamic balance between DNA methylation and demethylation, which is crucial for maintaining brain functions (Varley et al., 2013). Szwagierczak et al. (2010) showed that in the adult brain, among the members of the TET family, the TET3 enzyme is the most abundant member in the cerebellum, cortex, and hippocampus. These researchers further demonstrated that TET1 and TET3 have similar functions in controlling 5-hmeC levels in gene promoters (Szwagierczak et al., 2010). A study reported that, unlike TET1 and TET3, TET2 mainly regulates 5-hmeC levels in the gene body and not in the promoter region (Williams et al., 2011). Additionally, TET2 was found to protect the central nervous system from age-related deterioration by promoting the maintenance of hippocampal neurogenesis

and enhancing cognition in adult mice (Gontier et al., 2018).

Considering that our treatments significantly and distinctly modulated the expression of DNA-modifying enzymes, we hypothesized that the expression of RAs might be regulated via DNA methylation. Our results did not reveal significant differences in the correlation between the expression and the 5-meC/5-hmeC ratio of genes two genes studied. We found that LPS upregulated the expression of the *Adora2A* gene and significantly decreased the percentage of the 5-meC mark. In contrast, pretreatment with caffeine restored the percentage of the 5-meC mark to baseline values and negatively regulated the expression of the Adora2A gene only in the hippocampus (details in Supplementary Material). Studies on the methylation of adenosine receptors are limited, which partially complicates our conclusions. Only a single study reported that DNA methylation plays a role in the transcription of the Adora2A gene; however, this study was conducted in cell culture using a demethylating agent (Buira et al., 2010). Thus, we cannot exclude the possibility that DNA methylation is the driving force behind the transcriptional control of the Adora2A gene under inflammatory conditions in the CNS.

Regarding the methylation of the promoter region of the Adora1 gene, the presence of two CpG islands in the regulatory region makes it more difficult to analyze the results. Thus, conducting additional analyses to identify the region (CpG 1 or 2 island) where the transcription factor binding motif is located may help elucidate the effect of caffeine on the transcriptional control of the Adora1 gene in the hippocampus. Our results revealed that caffeine modulates the expression of the *Adora1* gene in the hippocampus, with the possible involvement of DNA methylation; these findings provide new insights into the field of neuroepigenetics.

The significant correlation found between the expression of Adora1 (A_1R) and Adora2A ($A_{2A}R$) genes under all conditions (Caffeine, LPS, and Caffeine + LPS) in the hippocampus and the striatum in the caffeine group raised new questions about the molecular mechanisms underlying this transcriptional control. In this context, the results of the bioinformatics analysis confirmed the importance of the presence of the supramolecular complex known as the Nrlp3 inflammasome in the Il 1β network and revealed new proteins that were not known to be related to the neuroprotective effect of caffeine. Two of them, including Esr1 and Ma pk13, were related to the gene *Adora1*, and the other two, including Arrb2 and Ma pk14, were related to the *Adora2A* gene. A study suggested that the downregulation of Esr1 signaling is essential for memory formation, and that activation of the hippocampal region impairs memory formation (Cho et al., 2015); this was also observed in our study but only in the hippocampus of those animals pretreated with caffeine. In the bioinformatics analysis, Adora2b was found to be a central hub of the connection between the *Adora1* and *Adora2A* gene networks. This gene is linked to timing events in cognitive dysfunction (Gile et al., 2020) and in synaptogenesis during the development of astrocytes (Tanaka et al., 2021). In this study, caffeine potentiated the effect of LPS on the two structures to different degrees.

StringDB is a biological database that focuses on protein-protein interactions. It compiles information from several sources to construct a comprehensive network of protein interactions. In StringDB, data from experimental studies, computational predictions, and existing databases can be integrated to construct a reliable map of protein associations across various organisms. In this study, the database was used to build interaction maps capable of broadening the scope of the relationships between the three proteins in a systemic panorama. However, although the database is large, the StringDB platform does not consider the possible diversity of genes in different populations around the world. Studies using consortiums, such as The International Genome Sample Resource (IGSR), can enrich this scenario by providing frequencies of alleles and haplotypes in different populations and possible effects on their molecular function. Although studies on the diversity of these genes are scarce, several studies have investigated the association between SNPs with various diseases (Szklarczyk et al., 2023). When using this methodology, different cell types can lead to different responses in gene expression. In this context, the interaction between these genes can be better evaluated using the scRNAseq model in future studies.

Overall, as shown in Fig. 7, our results showed that acute pretreatment with caffeine can differentially attenuate the deleterious effects of the inflammatory response in the CNS, especially in the transcriptional control of ARs between the striatum and the hippocampus; it can also downregulate *Il 1β*. A major limitation of our study was that we could not determine whether the modifications described occur in cells. However, our findings regarding the differential modulation of RAs (A_1R and $A_{2A}R$) between the hippocampus and striatum and the reduction in the LPS-mediated transcriptional activation of the gene encoding the pro-inflammatory cytokine *Il 1β* may help in designing future studies to better elucidate this complex network of interdependence, considering that such modifications might also occur in glial cells that control neuroinflammation, and neurons that regulate the effect of neuroinflammation.

4. Experimental procedure

4.1. Caffeine treatment and LPS-induced neuroinflammation

Adult male Swiss mice (n = 20; 2–4 months old; 45–50 g) were kept in polypropylene cages (32×40×18 cm), with a maximum of five animals per cage. The cages were kept in a room at 23±2 °C, and a 12-h/12-h light/dark cycle was maintained (lights were switched on at 7:00 a.m.). The humidity was maintained at 55–65 %, and a 3 cm layer of wood shavings served as a bed. All mice were provided water and food ad libitum during all experimental procedures. We excluded females from this study due to concerns about medical, genetic, psychosocial, and behavioral factors related to sex and gender and differences in neurodegenerative diseases (Aggarwal and Mielke, 2023) (Shi et al., 2024), along with hormonal factors (Cerri et al., 2019) that might interfere with the epigenetic mechanisms.

All animals were maintained in this controlled environment for 10 days before treatment to acclimate to laboratory conditions. Neuroinflammation was induced by a single intraperitoneal (i.p.) injection of *Escherichia coli* lipopolysaccharide (LPS – 0.33 mg/kg) (*E. coli* LPS, serotype 0127: B8 – Sigma-Aldrich – L3129). Caffeine (6 mg/kg) Sigma-Aldrich – C0750) was administered i.p. 15 min before administering LPS. All mice were euthanized 24 h after the LPS challenge, and the striatum and hippocampus were collected for molecular analyses. The mice were randomly divided into four groups (n = 5 animals per group): the **Vehicle** group – animals injected with 0.9 % NaCl (0.1 mL/10 g of body weight); the **Caffeine** group – animals that received caffeine (6 mg/kg of body weight); the **LPS** group – animals that received 0.33 mg/kg of body mass LPS; the **Caffeine + LPS** group – animals that received caffeine (6 mg/kg of body weight), followed by 0.33 mg/kg of body mass LPS after 15 min. The dosage of LPS administered was determined based on previous studies that demonstrated the neuroinflammatory effect of LPS (Ghisoni et al., 2015) (de Paula Martins et al., 2018).

4.2. RNA extraction and cDNA synthesis

For extracting total RNA, the hippocampus and the left striatum were collected 24 h after LPS was administered and immediately homogenized with the Ambion TRIzol Reagent (Life Sciences – Fisher Scientific Inc., Waltham, MA, USA). After homogenizing the samples in 0.5 mL of TRIzol®, the aqueous phase was separated by adding 0.2 mL of chloroform (Merck, Whitehouse Station, NJ, USA) and centrifuged at 14.000 rpm (Eppendorf/5804 R centrifuge) for 15 min at 4 °C. After centrifugation, the precipitate was discarded and the aqueous phase (supernatant) was collected in tubes. Then, the RNA was precipitated by adding 0.5 mL of ice-cold absolute isopropanol (Merck, Whitehouse Station, NJ, USA). Subsequently, the samples were incubated at room temperature for 15 min and centrifuged again at 14.000 rpm (Eppendorf/5804 R centrifuge) at 4 °C. After centrifugation, isopropanol was discarded by

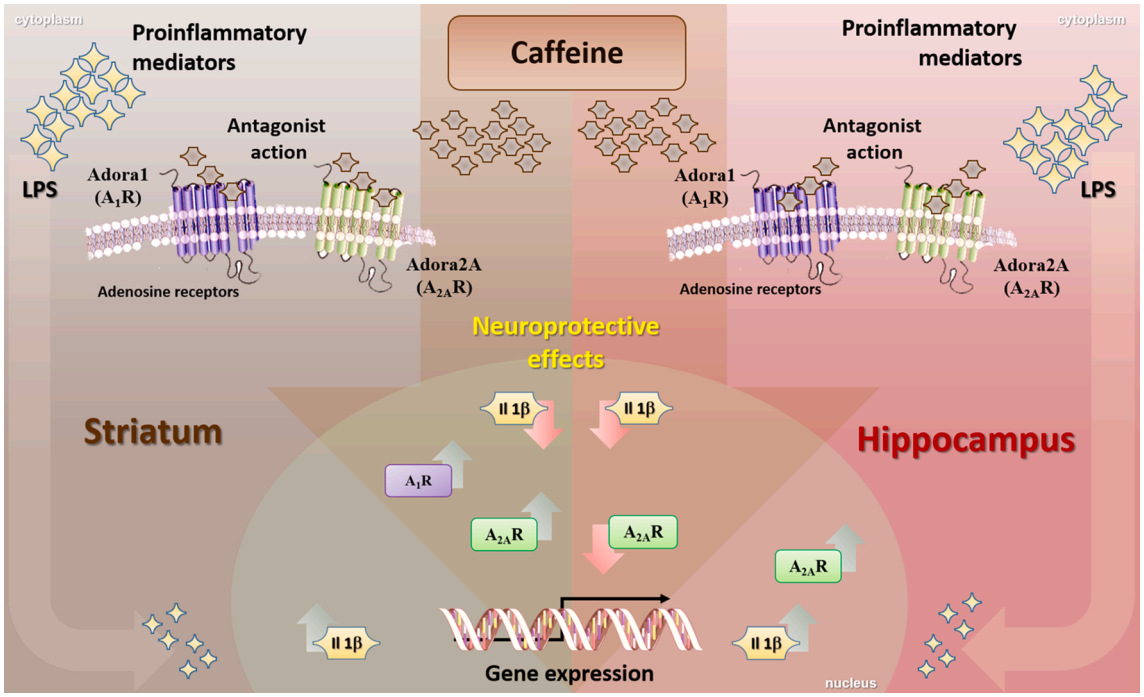

Fig. 7. Graphical abstract. Graphical representation of the main findings regarding acute caffeine treatment in a neuroinflammation model. Regarding gene expression, we found that caffeine decreased the expression of the Il 1β gene in the striatum and hippocampus, accompanied by an increase in the expression of the A_{2A} receptor in the striatum and a decrease in its expression in the hippocampus.

inversion, and the precipitated RNA (pellet) was washed with 75 % ethanol and resuspended in 20 mL of DEPC water. The quantity and purity of the RNA was estimated using a spectrophotometer, which measured the OD 260/280 ratios (≥1.8) and OD 260/230 ratios (≥1.0) (NanoDrop 2000, Thermo Scientific, Uniscience); the suitable RNA samples were stored at −80 °C. The cDNA was synthesized after the total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions.

4.3. Real-time reverse transcription and quantitative PCR (RT-qPCR)

We investigated the pattern of gene expression of the pro-inflammatory cytokine *Il 1β,* the enzymes involved in DNA methylation [DNA methyltransferase (*Dnmt1*), (*Dnmt3a*), and (*Dnmt3b*)] and DNA demethylation [dioxygenase-dependent 2-oxoglutarate (2OG) and iron II (*Tet1*), (*Tet2*), and (*Tet3*)], and the expression of the genes encoding adenosine receptors, *Adora1* and *Adora2A* by conducting RT-qPCR; all reactions were performed using specific primers. The reactions were conducted using a QuantStudio® 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, EUA), and the reaction mixture contained 10 μL (Syber Green Master Mix 2x − 5 μL, 0.4 μM) of each primer (for primers and conditions, see Table 1), 50 ng of cDNA, and nuclease-free H_2O. The quantity of mRNA present was corrected using the combination of three genes, including *18 s*, *β-actin,* and *Gapdh*, using the ΔΔCT method. All primers were synthesized by Exxtend Biotecnologia (Paulínia, São Paulo, SP, Brazil).

4.4. DNA extraction

For extracting genomic DNA (gDNA), the hippocampus and the left striatum were collected 24 h after LPS was administered and immediately homogenized in an extraction buffer (10 mM Tris pH 3.0; 0.5 % SDS, 5 mM EDTA) after digestion with proteinase K solution at 65 °C for 16 h. Next, 500 μL of equilibrium phenol was added to the tube, following which, the mixture was centrifuged at 12.000 rpm for 15 min at room temperature (Eppendorf/5804 R centrifuge). The upper aqueous layer containing the target DNA was preserved and mixed with 200 μL of chloroform (Sigma-Aldrich – 288306). The mixture was centrifuged at 12.000 rpm for 15 min (Eppendorf/5804 R centrifuge), and the supernatant was collected and transferred to a new tube. Then, 800 μL of isopropanol and 150 μL of 3 M sodium acetate (Sigma-Aldrich – W302475) were added to the mixture. The mixture was centrifuged again at 12.000 rpm for 15 min (Eppendorf/5804 R centrifuge). The supernatant was removed, and the pellet was washed with 500 μL of 70 % alcohol, followed by centrifugation at 12.000 rpm for 5 min. The supernatant was completely discarded, and 50 μL of nuclease-free H_2O was added to the tube. The quantity and purity was estimated using a spectrophotometer, which recorded the OD 260/280 ratios (≥1.8) and OD 260/230 ratios (≥1.0) (NanoDrop 2000, Thermo Scientific, Uniscience); the pure samples were stored at −20 °C.

4.5. Enzymatic gDNA treatment

After confirming the quantity and purity by spectrophotometry (OD 260/280 ≥1.8 and OD 260/230 ≥1.0), the gDNA was treated with T4-β-glucosyltransferase (T4-BGT), and subsequently, with *Msp*I and *Hpa*II (New England BioLabs, Beverly, MA, USA). For this, three tubes (A, B, and C) containing 400 ng of gDNA of each sample were treated with 40 mM UDP glucose and T4-BGT (1 unit) for 1 h at 37 °C. This enzyme catalyzes the transfer of the glucose moiety of uridine diphosphoglucose (UDP-Glc) to 5-hydroxymethylcytosine (5-hmeC) by a glycosylation reaction, which leads to the production of beta-glucosyl-5-hydroxymethylcytosine. This compound blocks the endonuclease activity of *Msp*I and promotes only the recognition of the methylated (5-meC) and unmethylated CCGG sequences, keeping the hydroxymethylcytosine (5-hmeC) CCGG regions unaltered. After the T4-BGT enzyme was inactivated by incubation at 65 °C for 15 min, the samples were digested with the endonuclease *Msp*I (New England Biolabs, Beverly, MA, USA), which can detect all CCGG sequences (methylated and unmethylated, except for the CCGG sequences with the product beta-glucosyl-5-hydroxymethylcytosine), and the enzyme *Hpa*II (New

Table 1
The oligonucleotide primers and PCR conditions used in quantitative real-time PCR.

Gene (ID)	Primer	5′- 3′ Sequence	Reactions Condicition	Product size (pb)
Il 1b (16176)	Forward	GAC CTT GGA TGA GGA CA	95 °C −15 s; 60 °C − 30 s; 72 °C − 30 s	183
	Reverse	AGC TCA TAT GGG TCC GAC AG		
Dnmt1 (13433)	Forward	CCT TTG TGG GAA CCT GGA A	95 °C −15 s; 63 °C − 30 s; 72 °C − 30 s	240
	Reverse	CTG TCG TCT GCG GTG ATT		
Dnmt3A (13435)	Forward	GAG GGA ACT GAG ACC CCA C	95 °C −15 s; 63 °C − 30 s; 72 °C − 30 s	216
	Reverse	CTG GAA GGT GAG TCT TGG CA		
Dnmt3B (13436)	Forward	AGC GGG TAT GAG GAG TGC AT	95 °C −15 s; 63 °C − 30 s; 72 °C − 30 s	91
	Reverse	GGG AGC ATC CTT CGT GTC TG		
Tet1 (52463)	Forward	GAG CCT GTT CCT CGA TGT GG	95 °C −15 s; 65 °C − 30 s; 72 °C − 30 s	367
	Reverse	CAA ACC CAC CTG AGG CTG TT		
Tet2 (214133)	Forward	AAC CTG GCT ACT GTC ATT GCT CCA	95 °C −15 s; 65 °C − 30 s; 72 °C − 30 s	211
	Reverse	ATG TTC TGC TGG TCT CTG TGG GAA		
Tet3 (194388)	Forward	GTC TCC CCA AGT CCT ACC TCC G	95 °C −15 s; 63 °C − 30 s; 72 °C − 30 s	137
	Reverse	GTC AGT GCC CCA CGC TTC A		
Adora1 (11539)	Forward	AGA ACC ACC TCC ACC CTT CT	95 °C −15 s; 63 °C − 30 s; 72 °C − 30 s	227
	Reverse	TAC TCT GGG TGG TGG TCA CA		
Adora2A (11540)	Forward	ATC CCT CAG AGA AGG GAA GC	95 °C −15 s; 63 °C − 30 s; 72 °C − 30 s	300
	Reverse	AGC TTC CCA AAG GCT TTC TC		
b-actin (11461)	Forward	TCT TGG GTA TGG AAT CCT GTG	95 °C −15 s; 58 °C − 30 s; 72 °C − 30 s	82
	Reverse	AGG TCT TTA CGG ATG TCA ACG		
Gapdh (14433)	Forward	AGG CCG GTG CTG AGT ATG TC	95 °C −15 s; 58 °C − 30 s; 72 °C − 30 s	530
	Reverse	TGC CTG CTT CAC CAC CTT CT		
18 s (19791)	Forward	CGC GGT TCT ATT TTG TTG GT	95 °C −15 s; 60 °C − 30 s; 72 °C − 30 s	179
	Reverse	TCG TCT TCG AAA CTC CGA CT		

England Biolabs, Beverly, MA, USA), which recognizes all CCGG sequences, with the difference that methylated CCGG sequences (5-meC) inhibit its catalytic activity. In the third tube, an equal quantity of H_2O was added (undigested gDNA – 100 % control). All reactions were conducted separately with a final volume of 25 μL at 37 °C for 16 h.

4.6. Methylation-specific qPCR (MS-qPCR)

The MS-qPCR methylation data were obtained from five animals and a technical duplicate. The pattern of methylation (5-meC) and hydroxymethylation (5-hmeC) of the promoter region of ***Adora1*** **(ID: 11539)** [island 1- chr1:134234720+134235068, 349 bp (F: 5‘ AAG GAG CTC ACC ATC CTG 3‘); (R: 5‘ GTG GGT GGG CAC AGG GTA G 3‘) and island 2 – chr1:134235378+134235645, 268 bp (F: 5‘ CGA GAC TCC ACT CTG GC 3‘); (R: 5‘ CAC CTC GGT ACT GTC CCT GT 3‘)] and ***Adora2A*** **(ID: 11540)** – chr10:75317069+75317671 603 bp (F: 5‘ AGG GTG CGC CCA TGA GCG GC 3‘); (R: 5‘ CAA CCC GAG AGT CTG ACC CGC CT 3‘) were determined by performing qPCR using reaction mixtures containing 2x SYBR Green I Master Mix (5 μL), 0.4 μM specific primers (1.0 μL), 25 ng of treated gDNA (1.5 μL – three conditions: H_2O, *Msp*I, and *Hpa*II), and q.s.p of nuclease-free H_2O (2.5 μL). Primer sequences were designed for regulatory regions with CpG islands within regions of hypersensitivity to DnaseI, regulated by histone modification markers and with transcription factor binding sites using the Primer3 Input program (version 0.4.0) (Untergasser et al., 2012). All primer sequences were blasted to confirm their chromosomal location using an in-silico PCR tool (https://genome.ucsc.edu/), and the secondary structures and annealing temperatures were evaluated using the Beacon Designer program (http://www.premierbiosoft.com/). The percentage of the 5-methylcytokine mark (5-meC) of the samples was determined based on the result of the difference between the CT obtained from the gDNA sample digested by the HpaII enzyme (sensitive to methylation) and the CT from the gDNA sample digested by the MspI enzyme (not sensitive to methylation), multiplied by 100 and divided by the Ct obtained from the intact gDNA sample (undigested – SE) [$(HpaII^{Ct} - MspI^{Ct})$ X 100/gDNA SE^{Ct}]. The determination of the percentage of the 5-hydroxymethylcytokine (5-hmeC) mark was determined by the difference between the Ct obtained from the gDNA sample digested by the MspI enzyme (not sensitive to methylation) and the Ct from the intact gDNA sample (not digested – SE) [$(MspI^{Ct}$ – gDNA SE^{Ct}] (Nestor et al., 2012).

4.7. Bioinformatics analysis

Expanded protein-protein interaction networks of the genes *Adora1*, *Adora2a,* and *Il1b* were constructed using StringDB (Szklarczyk et al., 2017). The active interaction sources included *Textmining*, *Experiments*, and Databases, and the minimum required interaction score was 0.700 (high confidence). The first shell contained 20 proteins, while the second shell contained 100 proteins. The R package *igraph* (version 1.2.11) was used to merge the three expanded networks into a single network and calculate the single paths (Csardi and Nepusz, 2006). A Venn diagram was constructed using the *ggvenn* (version 0.1.9) and *viridis* (version 0.5.1) packages (Garnier, 2018; Yan, 2021). Cytoscape (version 3.9) was used to visualize the merged network (Gustavsen et al., 2019) (Shannon et al., 2003). Enrichment analysis was performed using the clusterProfiler package (version 3.18.1), and the p-adjust threshold was considered to be 0.001 (Yu et al., 2012).

4.8. Statistical analysis

The differences between the variables (Caffeine and LPS) were evaluated by the two-way analysis of variance (ANOVA) test (Two-Way ANOVA), followed by Tukey’s post hoc test using the GraphPad Prism 7 program (GraphPad Software Inc., San Diego, CA, USA). All differences among and between groups were considered to be statistically significant at $P < 0.05$. All qPCR (expression) analyses were performed in technical duplicates using five animals per group.

Ethics approval

This study was ethically reviewed and conducted by ethical guidelines of the Ethics Committee for Animal Research (PP00760/CEUA) of the Universidade Federal de Santa Catarina, Florianópolis, Brazil.

Consent for publication

All the authors agree to the publication of this work.

Availability of data and material

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

CRediT authorship contribution statement

Paula Lemes dos Santos Sanna: Writing – original draft, Methodology, Funding acquisition, Formal analysis, Data curation. **Liebert Bernardes Carvalho:** Methodology, Formal analysis, Data curation. **Camila Cristina dos Santos Afonso:** Methodology, Formal analysis, Data curation. **Kassia de Carvalho:** Methodology, Formal analysis, Data curation. **Rogério Aires:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Jennyffer Souza:** Methodology, Formal analysis, Data curation. **Marcel Rodrigues Ferreira:** Writing – original draft, Supervision, Methodology, Formal analysis. **Alexander Birbrair:** Writing – review & editing, Methodology, Data curation. **Maria Martha Bernardi:** Writing – review & editing, Methodology, Investigation, Data curation. **Alexandra Latini:** Writing – review & editing, Investigation, Conceptualization. **Rodrigo A. Foganholi da Silva:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2024.148866.

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