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Caffeine inhibits hypoxia-induced nuclear accumulation in HIF-1α and promotes neonatal neuronal survival

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Abstract

Apnea of prematurity (AOP) defined as cessation of breathing for 15- 20 seconds, is commonly seen in preterm infants. Caffeine is widely used to treat AOP due to its safety and effectiveness. Caffeine releases respiratory arrest by competing with adenosine for binding to adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$). Long before its use in treating AOP, caffeine has been used as a psychostimulant in adult brains. However, the effect of caffeine on developing brains remains unclear. We found that A_1R proteins for caffeine binding were expressed in the brains of neonatal rodents and preterm infants (26–27 weeks). Neonatal A_1R proteins colocalized with PSD-95, suggesting its synaptic localization. In contrast, our finding on A_2R expression in neonatal neurons was restricted to the mRNA level as detected by single cell RT/PCR due to the lack of specific $A_{2A}R$ antibody. Furthermore, caffeine (200 μM) at a dose twice higher than the clinically relevant dose (36–130 μM) had minor or no effects on several basic neuronal functions, such as neurite outgrowth, synapse formation, expression of A_1R and transcription of CREB-1 and c-Fos, further supporting the safety of caffeine for clinical use. We found that treatment with $CoCl_2$ (125 μM), a hypoxia mimetic agent, for 24 hours triggered neuronal death and nuclear accumulation of HIF-1α in primary neuronal cultures. Subsequent treatment with caffeine at a concentration of 100 μM alleviated $CoCl_2$-induced cell death and prevented nuclear accumulation of HIF-1α. Consistently,

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caffeine treatment in early postnatal life of neonatal mice (P4-P7) also prevented subsequent hypoxia-induced nuclear increase of HIF-1α. Together, our data support the utility of caffeine in alleviating hypoxia-induced damages in developing neurons.

1. Introduction

Apnea of prematurity (AOP), defined as cessation of breathing for more than 20 seconds, is commonly seen in preterm infants (≤30 weeks of gestation). Preterm infants have a higher incidence of apnea due to exaggerated inhibition of the respiratory rhythm by adenosine signaling (Mathew, 2011). Methylxanthines, including caffeine, have been used to treat AOP for 40 years. Caffeine effectively releases this respiratory arrest by competing with adenosine for binding to adenosine receptors (Bairam et al., 1987). Clinically, relatively high concentrations of caffeine are used to treat AOP. The plasma concentrations of caffeine in preterm infants during AOP treatment were shown to be 10–100 times higher than that in infants receiving breast milk from mothers who drank moderate doses of coffee (Aden, 2011). The efficacy of caffeine was not evaluated until recently by a multicenter trial led by Erenberg and colleagues and by the international trial on caffeine for apnea (CAP trial) (Erenberg et al., 2000; Schmidt, 2005). The CAP trial suggested that caffeine is beneficial in lowering the incidence of several short-term morbidities, including bronchopulmonary dysplasia, and improving neurodevelopmental outcomes of premature infants at 18 to 21 months of age (Schmidt et al., 2007). These clinical trials not only validated the use of caffeine in alleviating AOP but also suggested that neonatal caffeine treatment may be beneficial to neurodevelopment in preterm infants.

Caffeine has been used to stimulate adult brains as a psychostimulant long before its use in treating AOP (Ferre, 2016; Frary et al., 2005). It is thought to modulate adult brain activity and plasticity by directly perturbing the purinergic system and indirectly affecting the transmission of other neurotransmitters (Fredholm et al., 1999). However, the effects of caffeine on developing brains remain unclear, especially when preterm infants receive such high concentrations of caffeine during AOP treatment.

Hypoxic ischemia is one of the major causes of early brain injury, which affects around 0.1–0.3% of term infants and the incidence in preterm infants is 0.1–0.8% (Davidson et al., 2018). Therapeutic strategies to treat early brain injury include neuroprotective agents and plasticity enhancing agents for functional recovery. Caffeine has been suggested to have both effects: 1) caffeine has been suggested to be neuroprotective in both rodents and preterm infants (Back et al., 2006; Cunha, 2001; Ferreira and Paes-de-Carvalho, 2001; Fredholm et al., 2011; Rivkees and Wendler, 2011; Winerdal et al., 2017) (Schmidt et al., 2007) and 2) caffeine is known to modulate plasticity in adult brains (Fredholm et al., 1999). We therefore propose that caffeine is a candidate agent for alleviating the damages caused by hypoxia ischemia in neonatal brains.

Adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) are the primary targets of caffeine (Fredholm et al., 1999). Thus, characterizing the expression of these receptors in neonatal brains is essential before testing the effects of caffeine for treating early brain injury. A_1R and $A_{2A}R$ can be detected in rat brains at perinatal stages by *in situ* hybridization and

radioactive ligand binding assays, albeit at lower levels compared to adult brains (Aden et al., 2000; Aden et al., 2001; Rivkees, 1995). However, the protein expression and subcellular localization of these receptors in the brain have not been identified in neonatal rodents and preterm infants. Using an antibody specifically against A_1R in wild type mice, but not in A_1R deficient mice, we found that A_1R displayed a punctate staining pattern and were synaptically localized in the brains of both neonatal mouse pups (postnatal day 3) and preterm infants (corrected age of 26–27 weeks). In contrast to A_1R, the localization of $A_{2A}R$ in neonatal brains remains unclear due to the poor specificity of tested antibodies against $A_{2A}R$. Furthermore, we also demonstrated the safety and therapeutic effects of caffeine on neonatal neurons. We found caffeine at a concentration of 200 μM did not affect basic neuronal functions, such as 1) expression and distribution of adenosine receptor, A_1R, 2) mRNA levels of transcription factors, CREB-1, c-Fos and HIF-1α, and 3) neurite outgrowth and synapse formation. Importantly, we found that at a clinically relevant dose, caffeine promoted survival of primary neurons after hypoxic insults and prevented hypoxia-induced accumulation of HIF-1α both in primary culture (100 μM of caffeine) and in neonatal mouse pups (estimated serum concentration of caffeine is 26–77 μM).

2. Materials and Methods

The protocols for animal experiments were approved by IACUC at SUNY Downstate Medical Center, Rutgers University and Feinstein Medical Institute. The use of autopsy specimens of preterm brain tissue from the neuropathology laboratory was approved by IRB at Cohen Children's Medical Center.

2.1. Primary neuronal cultures

Primary neuronal cultures were prepared from E18 or P8 rat pups as described. Briefly, the hippocampus, cerebellum or cortex was dissected followed by trypsinization and centrifugation. The pellet was then resuspended in neuronal culture medium (Neurobasal medium supplemented with 2% B27 and 25 mM KCl) and seeded at a density of 5×10^5 cells/ml (for RT/PCR and immunostaining) or 1×10^5 cells/ml (for MTT assays).

2.2. Induction of hypoxia *in vitro*

Cobalt chloride ($CoCl_2.6H_2O$) (Sigma) was used to induce hypoxia in primary neuronal cultures as previously described (Wu and Yotnda, 2011). Five-day old neuronal cultures were treated with various concentrations of $CoCl_2$ (50–125 μM) for 24 hours. After washing off the $CoCl_2$, cultures were replenished with conditioned medium which was supplemented with 100 μM caffeine or vehicle control for an additional 24 hours, followed by fixation and analysis.

2.3. Neonatal hypoxia and caffeine treatment *in vivo*

Mouse pups were given caffeine at a loading dose of 20 mg/kg/day on postnatal day 4 (P4), then 15 mg/kg/day for the next 3 days. Caffeine was dissolved in sterile water and given to mouse pups via orogastric tube 1.9 french to maintain a serum concentration of caffeine at 5–15 mg/l (approximately 26–77 μM) (Gaytan et al., 2006; Guillet and Kellogg, 1991). Sham groups were given equal volumes of sterile water via the orogastric route. In both

groups, half of the pups were exposed to hypoxic environment (8% oxygen) for 20 min on P7, whereas the other half remained in normoxia. Pups were then deeply anesthetized with a lethal dose of xylazine (30mg/kg)/ketamine (300mg/kg) and perfused transcardially with 4% PFA.

2.4. Immunostaining

Immunostaining was performed using antibodies against A_1R or $A_{2A}R$ (Antibody Online, GA and Genetex, CA and AbCam, MA), PSD-95 (Santa Cruz, CA), beta-III tubulin (Sigma), AMPA, CREB-1 and syntaxin (all from Genscript, NJ), HIF-1α (Novus, CO, Genetex, CA and AbCam, MA) and c-Fos (Gentex, CA and Biorbyt, CA). The immune complexes were detected by FITC-conjugated anti-mouse antibodies or Cy3-conjugated goat anti-rabbit antibodies (Jackson Immunoresearch, PA). The resulting images were acquired using Axiovert fluorescent microscopy (primary neuronal cultures), Zeiss LSM710 (mouse brains) or Olympus Fluoroview 300 (preterm infant brains) confocal microscopy. The brains of mice deficient in A_1R or $A_{2A}R$ were kindly provided by Dr. William Welch (Georgetown University) and Dr. Bruce Cronstein (NYU), respectively.

Unless specified, all mouse brains were immunostained using vibratome sections (50 μm thick). For A_1R and PSD-95 co-localization experiments, cryosectioned slices were used. For study of the expression of A_1R in postmortem preterm human brain, tissues were collected from the autopsy specimens of preterm infants (gestational age of 23–24 weeks) who were given caffeine on the first day of life and continued until death at three weeks of life. These tissues were fixed in 10% formalin, paraffin embedded and sectioned coronally at 6 μm thick. Following antigen retrieval, sections were incubated with A_1R antibody.

The densitometries of single cell RT-PCR bands and the neurite length in the acquired images of beta-III tubulin immunostaining were quantified using NIH ImageJ. The number of A_1R, syntaxin and AMPAR puncta was quantified using the Analyze Particle function of Fiji ImageJ (size=30–250, circularity=0.3–1.0) as previously described (Muller et al., 2018; Taylor et al., 2010). The pixel intensity of HIF-1α in the nucleus and cytoplasm was measured using ImageJ.

2.5. MTT viability assay

Primary neurons were seeded at a concentration of 1×10^5 cells/ml and cultured for 7 days prior to the addition of 125 μM of $CoCl_2$ for an additional 24 hours. $CoCl_2$ containing medium was then replaced with conditioned medium in the presence or absence of 100 μM caffeine for an additional 24 hours. Neuronal viability was tested using the MTT assay as follows.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to medium at a final concentration of 0.5 mg/ml and incubated with the cells at 37°C for 2 hours. The resulting purple crystals (due to the action of mitochondrial reductase) were solubilized with DMSO and the optical density at 570 nm was subsequently measured.

2.6. Single cell RT-PCR

Glass cover slips coated with poly-L-lysine (Sigma) were cut at dimensions of approximately 2×3 mm. These cover slips were placed in 24 well tissue culture plates. Primary neurons were then seeded at a density of 5×10^5 cells/ml. After 48 hours, caffeine was added to the medium for an additional 5 days. Based on the morphology, cover glasses containing single neurons were harvested for RT-PCR as previously described (Dulac and Axel, 1995; Li et al., 2009). Reactions that were positive for the expression of GFAP, a glial cell marker, were discarded. All PCR products were subjected to sequencing analysis. The primers used to amplify the corresponding fragment of the genes are listed in the following table.

Gene (Gene Bank No.)	Primers
Adenosine A_1 Receptor (NM64299)	F: ACC TGC CTC ATG GTG GCC TG R: GTA GTA CTT CTG GGG GTC ACC G
Adenosine A_{2A} Receptor (AF2228684)	F: GGA GCT GGC CAT CGC TGT GC R: TCG CCG CAG GTC TTC GTG GA
Actin (NM031144)	F: CCC TGT GCT GCT CAC CGA GG R: GCG GCA GTG GCC ATC TCT TG
CREB-1 (NM_031017.1)	F: GGG CAG ACA GTC CAG GTC CAT R: GCC AGC TGT ATT GCT CCT CCC
C-Fos (NM_022197)	F: ACG AGG CGT CAT CCT CCC GCT R: CCC TCC TCC GAT TCC GGC AC
HIF-1α (NM_24359.1)	F: GCA GCA CGA TCT CGG CGA AGC R: CAT CGT CCT CCC CCG GCT TG

2.7. Statistics

Data were analyzed using GraphPad Prism 7 software (GraphPad Software Inc., CA.). Student's *t* test was used to compare the data of two experimental groups, whereas the data of more than two groups were analyzed by ANOVA. The values of $p < 0.001$ were considered statistically significant. All data are shown as mean ± SEM.

3. Results

3.1. Adenosine A_1 receptors are synaptically localized in neonatal brains

We first asked whether the brains of both neonatal mice and preterm infants express the primary receptors for caffeine, adenosine receptors A_1R and $A_{2A}R$ by immunostaining. We chose immunostaining instead of Western blotting because Western blotting does not show the subcellular localization of these receptors and the tissue extracts often include non-neuronal cell types.

We screened the specificity of several commercially available antibodies against A_1R and $A_{2A}R$. We chose antibodies that showed high immunoreactivity in wild type mouse brains but not in the brains of the corresponding knockout mice. Using two antibodies against different epitopes of A_1R, we consistently observed A_1R distributed in a punctuate pattern in

adult wild type brains (25.33±2.40 puncta), but not in A_1R-deficient brains (8.67±0.88 puncta, $p<0.001$) (Figure 1A, top panel; enlarged images are in Supplementary Figure S1A).

In contrast to A_1R, several commercially available antibodies against $A_{2A}R$ displayed low specificity, showing intense nuclear staining in both wild type and $A_{2A}R$ knockout brains (Figure 1A, bottom panel; enlarged images are in Supplementary Figure S1A). Thus, this study focused mainly on A_1R, but not $A_{2A}R$, in neonatal brains.

Similar to adult brain tissues, we found A_1R also distributed in a punctate pattern in neonatal cortex (Figure 1B). This punctate pattern can be detected in neonatal mouse cortex as early as postnatal day 3 (P3), albeit the puncta are smaller in size compared to those in older pups (P10 and 1 month old) (Figure 1B; an enlarged image of P3 brain is in Supplementary Figure S1B). In addition to cortex, A_1R was also expressed in a similar punctate pattern in various brain regions, such as medulla, cerebellum, thalamus and hippocampus (Figure 1C; an enlarged image of medulla is in Supplementary Figure S1C). A_1R puncta were most prominent in medulla and cerebellum (Figure 1C), but weak in basal ganglia (data not shown). The punctuate pattern of A_1R is reminiscent of that of synaptic markers. Using double immunostaining, we found that A_1R co-localized with PSD-95, a synaptic protein, suggesting A_1R is synaptically localized in neonatal mouse brain (the earliest time point we studied is P3) (Figure 1E, top panel; an enlarged image is in Supplementary Figure S1E).

We next sought to extend our findings to postmortem preterm brains. Most deceased preterm infants in our clinics were born at approximately gestational age of 23–24 weeks and died at the corrected age of 26–27 weeks. Among these patients, we selected infants with no discernible brain abnormalities on head ultrasound. It is worth noting that all selected infants received caffeine since it is our policy that all preterm infants with gestational age younger than 29 weeks be given caffeine within hours after birth. We therefore did not have age-matched preterm infants who did not receive caffeine among this age group. However, we still found detectable levels of A_1R puncta in these preterm brains, as demonstrated in the cortex of 6 different preterm infants (a representative section of each patient is shown in Figure 1D; an enlarged image is in Supplementary Figure S1D). These A_1R puncta co-localized with PSD-95 (Figure 1E, bottom panel). Together, these data supported the hypothesis that the preterm brain may be influenced by caffeine during therapy to treat AOP.

3.2. Caffeine promotes cell survival and suppresses hypoxia-induced nuclear accumulation of HIF-1α *in vitro*

Caffeine has been suggested to be neuroprotective, but its safety and effectiveness in neonatal neurons remained unclear (Aden, 2011; Natarajan et al., 2007; Rivkees and Wendler, 2011; Schmidt et al., 2007; Winerdal et al., 2017). Here, we sought to address these issues both *in vitro* and *in vivo*. When investigating whether caffeine perturbs the general neuronal functions, we used a higher concentration of caffeine (200–400 μM). However, we returned to the clinically relevant doses of caffeine (50–100 μM) to evaluate its neuroprotective effects on primary neurons after ischemic insults.

We examined three neuronal aspects to gauge the safety of caffeine: 1) expression of A_1R and $A_{2A}R$, 2) levels of several transcription factors, CREB-1, c-Fos and HIF-1α, and 3)

synapse formation and neurite outgrowth. First, we analyzed the effects of caffeine on the transcription of A_1R and $A_{2A}R$ by single cell RT/PCR as well as their protein levels by immunostaining. To ensure specificity, all RT/PCR products were sequenced to verify the identity of adenosine A_1R and $A_{2A}R$. The intensities of the PCR products were quantified using the densitometry function of ImageJ in arbitrary units. We found that neonatal neuronal cultures expressed mRNA for both adenosine A_1R and $A_{2A}R$ in untreated controls (2104±138 and 2446±130). With 200 μM caffeine treatment, the transcription of A_1R and $A_{2A}R$ was comparable to that of untreated neurons (2000±136 and 2500±150). However, when the concentration of caffeine was raised to 400 μM, caffeine selectively impaired the transcription of A_1R, but not A_2R (360±0.3 and 2280±77) (Figure 2A). Second, caffeine treatment was suggested to regulate the expression of several transcription factors, such as c-Fos, CREB-1, and HIF-1α (Connolly and Kingsbury, 2010; Gaytan and Pasaro, 2012) (Maugeri et al., 2018; Merighi et al., 2007). CREB-1 and c-Fos are immediate early genes induced by neural activity, whereas HIF-1α is often induced by hypoxic conditions. We examined the effects of caffeine on these transcription factors in neuronal cultures under normoxic conditions using single cell RT/PCR. In the absence of caffeine, there was low baseline expression of c-Fos but higher levels in HIF-1α and CREB-1 mRNA (HIF-1α, 2978±17.5; CREB-1, 2928±64; c-Fos, 105±7.5) (Figure 2A). With treatment of either 200 μM or 400 μM caffeine, we did not observe any significant changes in the transcription of HIF-1α, CREB-1 and c-Fos (HIF-1α, 3141±193 and 3095±111; CREB-1, 2933±88 and 3007±64; c-Fos, 132±7.6 and114±4) (Figure 2A). Third, beta-III tubulin and syntaxin are commonly used markers for the measurement of neurite length and synaptogenesis (Katsetos et al., 2003; Shin, 2014). We found that the average neurite length (162.1±17.45 μm) and the number of syntaxin puncta (15.33±1.76) in neurons that were treated with 200 μM caffeine for 5 days is comparable to that of the untreated neurons (184.9±27.6 μm and 14.67±1.45, respectively) (Figure 2B). Together, our data defined the safety threshold for caffeine at 200 μM since this dose did not perturb basic neuronal functions and characteristics *in vitro*.

We next sought to determine whether caffeine treatment promotes neuronal survival after hypoxic ischemia. Many of the cellular responses induced by hypoxia are mediated by changes in gene expression, which are often regulated by HIF-1α (Ke and Costa, 2006; Semenza, 2007; Shi, 2009). Under normal conditions, HIF-1α is constitutively degraded by prolyl-hydroxylase enzymes. However, hypoxia stabilizes HIF-1α by inhibiting these enzymes. Cobalt chloride ($CoCl_2$) is a hypoxia mimetic agent *in vitro* since it also inhibits prolyl-hydroxylase enzymes, resulting in accumulation and nuclear translocation of HIF-1α (Li et al., 2015; Piret et al., 2002). Here, we used $CoCl_2$ to mimic hypoxia *in vitro*. Primary neurons (5–7 days in culture) were treated with 50–125 μM $CoCl_2$ for 24 hours as previously described (Wu and Yotnda, 2011). The culture medium was discarded and replenished with conditioned medium in the presence or absence of 100 μM caffeine for an additional 24 hours. Subsequently, cells were subjected to MTT assay to measure cell survival rate (Denizot and Lang, 1986). In this experiment, the MTT reading of control group (no treatment) was defined as 100% viability. The MTT readings of other groups were compared with that of the control group, and the percentage of viability was derived accordingly. We found that the viability of neuronal cultures was comparable in the presence or absence of caffeine (Figure 3A; control, 100%; caffeine alone, 94±7.1%), suggesting

caffeine does not affect baseline cell survival. As expected, $CoCl_2$ decreased the viability of neuronal cultures compared to that of untreated cultures (Figure 3A; $CoCl_2$ treatment, 65.6±3.6%, p<0.001). Interestingly, the low viability caused by $CoCl_2$ was alleviated significantly by subsequent treatment with 100 μM caffeine (Figure 3A; $CoCl_2$+caffeine, 87.2±10.5%, n=4. p<0.001).

HIF-1α appears to have complex effects in hypoxic brains in a time-dependent manner (Supplementary Table S1). Accumulation of HIF-1α after ischemia has been shown to facilitate cell death at the very early phase (<24 h, pro-death effect) (Barteczek et al., 2017; Cheng et al., 2014; Yang et al., 2017), but HIF-1α has also been shown to limit infarct size at a later stage (>4 d, pro-survival effect) (Baranova et al., 2007; Bergeron et al., 2000; Sheldon et al., 2014; Sheldon et al., 2009). Since caffeine treatment increased neuronal viability immediately after hypoxia, we hypothesized that caffeine could decrease the accumulation of HIF-1α, therefore inhibiting its pro-death effect. To test this hypothesis, neuronal cultures were treated as in Figure 3A. We then co-immunostained the neuronal culture with 1) antibody against beta-III tubulin for neuronal labeling, 2) antibody against HIF-1α transcription factor and 3) DAPI for nuclear staining. The ratio of the pixel intensity of HIF-1α immunostaining in the nucleus vs cytoplasm of the neurons was computed using Image J. Prior to the induction of hypoxia, the ratio of nuclear vs cytoplasmic HIF-1α is comparable in cultures treated with 100 μM caffeine or vehicle (0.78±0.03 and 0.73±0.07, respectively) (Figure 3B and 3C, Lane 1 and 2), suggesting that caffeine alone did not affect nuclear levels of HIF-1α. However, this ratio increased significantly in $CoCl_2$-treated cultures (1.81±0.17, p<0.001) compared to that of untreated cultures, suggesting nuclear accumulation of HIF-1α in $CoCl_2$-induced hypoxia (Figure 3B and 3C). For most of the study, we used 125 μM $CoCl_2$ since this concentration triggered a significant nuclear accumulation of HIF-1α and cell death (Figure 3B and 3C). Interestingly, subsequent treatment with 100 μM of caffeine alleviated this hypoxia-induced nuclear accumulation of HIF-1α (0.82±0.04, p<0.001) (Figure 3B and 3C).

3.3. Caffeine suppresses hypoxia-induced nuclear accumulation of HIF-1α factor *in vivo*

We next sought to examine whether caffeine treatment in newborn mice is neuroprotective for subsequent hypoxia exposure. Here, caffeine was given to neonatal pups before induction of hypoxia in order to 1) avoid the high mortality rate when carotid ligation is performed in newborn mice at the age of P0-P4, and 2) avoid the injury-induced plasticity obscuring the potential effects of caffeine on neuroprotection and 3) mimic the timing of caffeine therapy in preterm infants.

Neonatal pups were gavage fed with caffeine as previously described (Gaytan et al., 2006). After caffeine treatment, the animals were exposed to a hypoxic or normoxic environment for 20 min before euthanization (Zaghloul et al., 2014). Their brains were removed immediately after the procedure and the brain sections were immunostained with HIF-1α antibody and counter stained with DAPI.

We first ensured caffeine treatment did not perturb basic neuronal functions. We found that the expression and subcellular localization of A_1R in various brain areas were similar whether neonatal pups received caffeine or not (Figure 4A, the puncta number in cortex is

70±1.15 in control group and 67.3±1.86 in caffeine group). Caffeine treatment also did not affect the punctate pattern of A_1R in cerebellum and brain stem (data not shown). In addition, we did not observe any significant changes in immunostaining for AMPA receptors, CREB-1 and c-Fos in the brains of neonatal rodents that were treated with vehicle or caffeine (AMPAR puncta, 67.6±2 and 63.6±2; CREB-1, 79±2.1, 81.3±1.5; c-Fos, 1±0.58, 1±0.1, respectively) (Figure 4A; an enlarged image of AMPA receptor puncta is in Supplementary Figure S3). Together, these data suggested that this clinically relevant dose of caffeine did not have a discernible impact on A_1R expression and the protein levels of AMPAR, CREB-1, and c-Fos.

Under normoxia, caffeine treatment did not affect the ratio of nuclear vs cytoplasmic HIF-1α in the brain of P7 pups whether the animals received caffeine or not (0.71±0.05 for control group and 0.77±0.03 for caffeine treatment group) (Figure 4B and 4C, Lane 1 and 2). Hypoxia increased the ratio of nuclear vs cytoplasmic HIF-1α in P7 pups that did not receive caffeine therapy (1.33±0.05, $p<0.001$) (Figure 4B and 4C, Lane 3). In contrast, the same hypoxic condition failed to trigger a significant nuclear accumulation of HIF-1α in neonatal pups that had received caffeine feeding previously (the ratio of nuclear vs cytoplasmic HIF-1α is 0.9±0.02, $p<0.001$) (Figure 4B and 4C, Lane 4), suggesting a prophylactic effect of caffeine in preventing upregulation in HIF-1α by subsequent hypoxia.

4. Discussion

Caffeine therapy is widely used to treat AOP because of its effectiveness and safety. Caffeine relieves AOP by competing with adenosine for binding to its receptors, A_1R and $A_{2A}R$, in brain stem. However, the effects of caffeine at this high dose on neonatal brains remained unclear. In this study, we first showed that the neonatal brain of both rodents and preterm infants expressed A_1R and that this receptor was enriched in synapses. In addition, we also detected $A_{2A}R$ mRNA by single cell RT/PCR in primary neuronal cultures. These data suggested that neonatal brains could be responsive to caffeine via A_1R and potentially $A_{2A}R$. We then showed that caffeine was considered safe in neonatal neurons since it did not perturb several basic neuronal functions, including the expression and distribution of A_1R, transcription of CREB-1, c-Fos and HIF-1α and neurite outgrowth and synapse formation. Lastly, we determined the beneficial effects of caffeine in alleviating hypoxia-induced neuronal death. We found that caffeine at clinically relevant doses suppressed hypoxia-induced nuclear accumulation of HIF-1α both *in vitro* and *in vivo*, and promoted viability in neuronal cultures exposed to hypoxia.

4.1. A_1R is synaptically localized in neonatal brains

The major receptors for caffeine are adenosine A_1R and $A_{2A}R$ (Aden et al., 2000; Fredholm, 1995). However, the protein expression and subcellular localization of these receptors in developing neurons remain unclear. We found that the brains of neonatal mice and deceased preterm infants expressed A_1R in brain areas, such as cortex, basal ganglia, thalamus, and hippocampus. We also found A_1R was enriched in synapses. The synaptic targeting of A_1R began at neonatal stages, albeit to a lesser extent, since the A1R puncta became more prominent as the mouse pups matured into adulthood (Figure 1B). Interestingly, the synaptic

targeting of A_1R is functionally linked to its neuromodullatory role in the adult brain (Rebola et al., 2003; Sheth et al., 2014).

In contrast to A_1R, we found $A_{2A}R$ mRNA by single cell RT/PCR was expressed in neonatal neuronal culture, but the protein distribution of $A_{2A}R$ in neonatal brains remained unclear. Previous reports showed soma enrichment of $A_{2A}R$ in the respiratory nuclei of neonatal brain stem (Gaytan and Pasaro, 2012). We also observed soma/nuclear enrichment of $A_{2A}R$ by immunostaining, but unfortunately, this strong immunostaining was present in both wild type brains and $A_{2A}R$ deficient brains, rendering inconclusive results (Figure 1A). As a control, we also tested these antibodies in adult brains since radio-ligand binding assays have clearly shown that the expression of $A_{2A}R$ is more enriched in striatum and olfactory bulb in adult brains (Fredholm et al., 2011), but none of these commercial antibodies showed this immunostaining pattern in adult brains.

4.2. Clinically relevant doses of caffeine had minor/no effects on synapse formation and expression of A_1R, CREB-1 and c-Fos

Caffeine has been shown to regulate the synaptic functions in adult cortical regions in several *in vitro* experiments (Greene et al., 1985; Yoshimura, 2005). However, we did not observe any differences in neurite length, synaptic density or the expression of several transcription factors between caffeine-treated and untreated neonatal neuronal culture. Moreover, under normoxic condition, the morphology of neonatal brains from pups receiving caffeine treatment appeared similar to that from untreated group.

A previous study has shown that caffeine increased A_1R transcription in the brain stem and the respiratory nuclei of the hypothalamus when caffeine was given to neonatal pups directly (Gaytan and Pasaro, 2012). The same study also demonstrated a caffeine-induced early shift of the postnatal increase in c-Fos in these regions. However, in our study, we did not observe any changes in 1) the protein levels of A_1R in the neonatal cortex and other regions (hippocampus/medulla/cerebellum/thalamus), and 2) the transcription levels of A_1R in neuronal cultures. We also found that the protein levels of c-Fos were low in both control and caffeine-treated groups. Since we also fed neonatal pups with caffeine directly or added caffeine into neuronal cultures directly, this discrepancy may arise from analyzing different brain regions and using different methodology. For example, we used single cell RT-PCR to analyze pure neuronal population, whereas Gaytan and colleagues used qPCR to analyze the whole extracts of hypothalamus and respiratory nuclei.

4.3. Caffeine blocks hypoxia-induced nuclear accumulation in HIF-1α and promotes neuronal survival

It has been suggested that caffeine has opposite effects on embryonic vs neonatal brains because caffeine is a potent inhibitor of A_1R. During the embryonic stage, inhibition of A_1R rendered cells more susceptible to hypoxia, whereas inhibition of A_1R in neonatal brains after birth was neuroprotective in white matter injury and hypoxic ischemia in rodents (Rivkees and Wendler, 2011). Notably, caffeine therapy for AOP and most related research in neonatal rodents employed postnatal treatment. Thus, caffeine has been suggested to have neuroprotective effects during the neonatal period (Back et al., 2006; Cunha, 2001; Ferreira

and Paes-de-Carvalho, 2001; Fredholm et al., 2011; Rivkees and Wendler, 2011; Winerdal et al., 2017) and are beneficial to neurodevelopment in preterm infants (Murner-Lavanchy et al., 2018; Schmidt et al., 2007).

Here, we showed a novel mechanism by which caffeine exerts its neuroprotective effects in neonatal brains. Hypoxia generally results in tissue damage immediately after ischemia. The "pro-death" effect of HIF-1 at this stage has been shown to facilitate death of severely injured neurons (Supplementary Table S1) (Barteczek et al., 2017; Cheng et al., 2014; Liu et al., 2015; Yang et al., 2017). We found that caffeine alleviated hypoxia-induced accumulation of HIF-1α both *in vivo* and *in vitro* and therefore increased the viability of primary neurons. This inhibitory effect is specific to nuclear accumulation of HIF-1α, not on its transcription (Supplementary Figure S2). In addition, caffeine had neuroprotective effects *in vivo* whether caffeine was administered before or after hypoxic insults. A recent study showed that a single dose of caffeine (5 mg/ml via intraperitoneal injection) immediately after neonatal hypoxic ischemia in neonatal pups decreased brain atrophy and improved behavioral outcomes at 2 weeks after hypoxic insults (Winerdal et al., 2017). However, in our study, caffeine was given to neonatal pups (P4-P7) before induction of mild hypoxia (8% O_2 in 20 minutes). We found that early caffeine therapy could prevent subsequent hypoxia-induced accumulation of HIF-1α. Clinically, our data suggest that caffeine therapy in the NICU may protect preterm brains from hypoxic damages that may occur during their NICU stay.

In conclusion, our results further support the utility of caffeine in preventing hypoxia-induced nuclear accumulation of HIF-1α and promotes neuronal survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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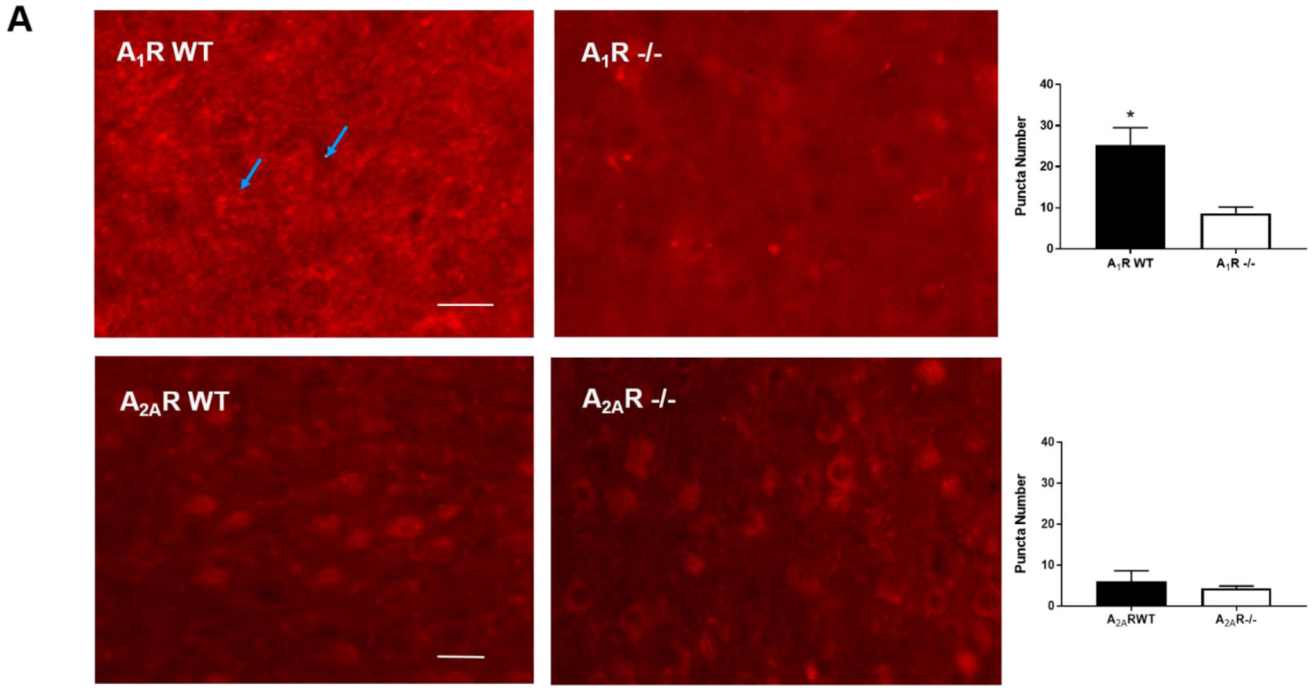
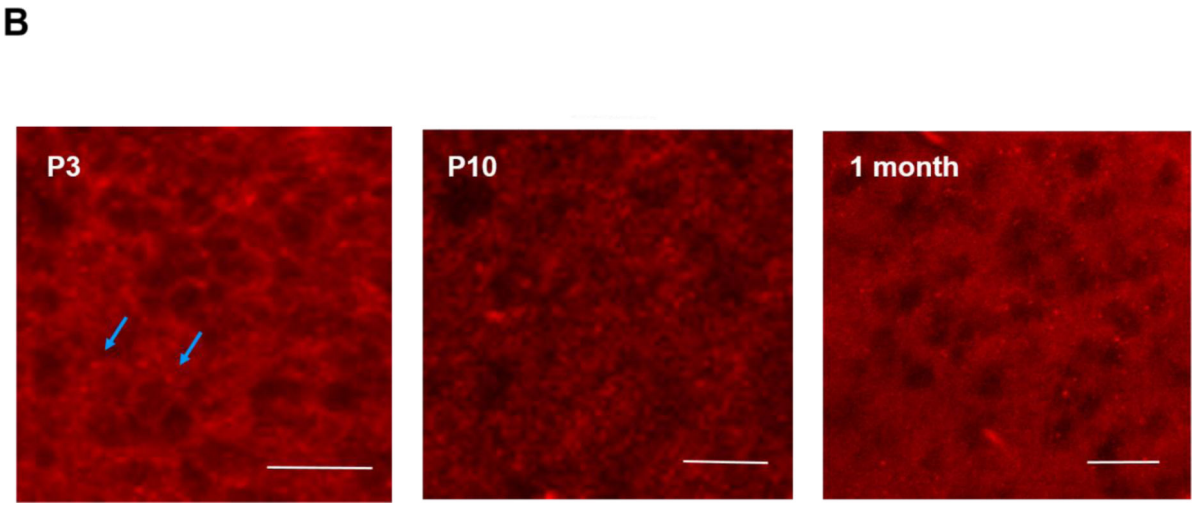
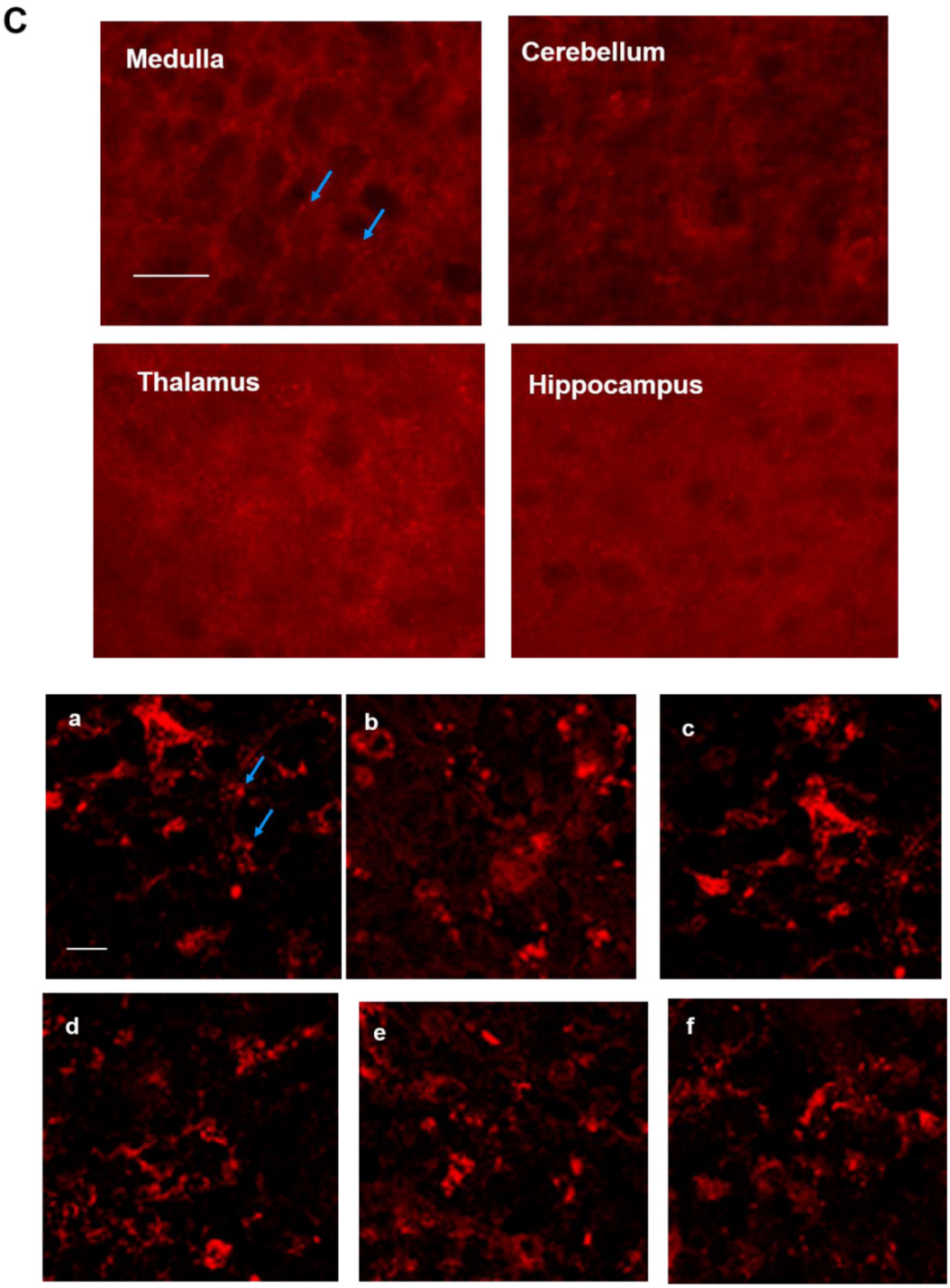
Highlights

- Neonatal brains express adenosine A1R in synapses for caffeine binding.
- Caffeine treatment does not affect the basal neuronal function.
- Caffeine promotes cell survival and inhibits hypoxia-induced accumulation of HIF-1α.







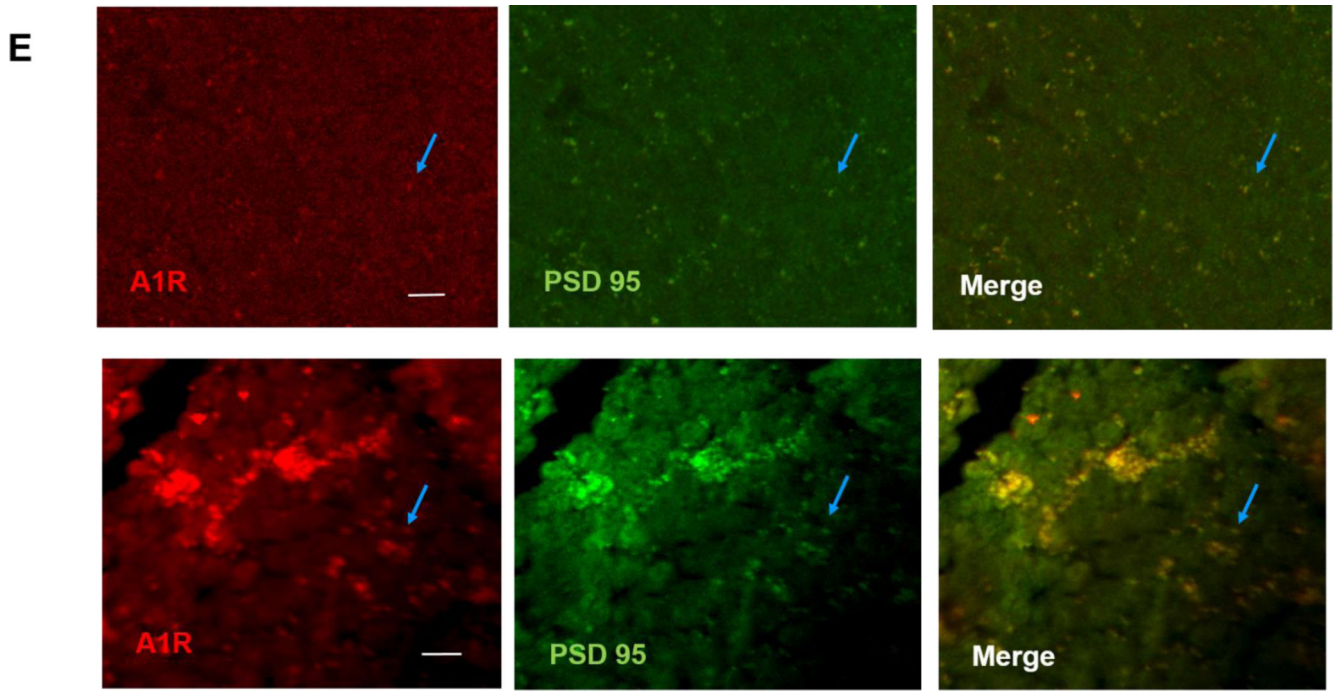


Figure 1.

The brains of neonatal mice and preterm infants expressed A_1R receptors. (A) Immunostaining results showed the specificity of A_1R antibody but not $A_{2A}R$ antibody. Using the tested A_1R antibody, A_1R immunostaining showed a punctate distribution of this receptor in the cortex of wild type adult mice, but not in that of A_1R deficient mice (25.33±2.4 and 8.67±0.88 puncta, respectively) (top). In contrast, several antibodies against $A_{2A}R$ showed non-specific immunoreactivity in the nucleus and the cytoplasm in both wild type and $A_{2A}R$ deficient mice (bottom). No punctate pattern was observed (6±1.53 puncta for WT and 4.33±0.3 puncta for $A_{2A}R$ knockout). Representative images are shown here. The quantitative results are shown on the right. Blue arrows indicate puncta. Scale bar, 10 μm. (B) The punctate pattern of A_1R could be detected in the cortex of P3 pups. The A_1R puncta appeared more discrete at a lower magnification as the animals mature (10 day-old and 1 month-old pups). (C) A_1R distributed in a punctate pattern in medulla, cerebellum, thalamus and hippocampus. The puncta were more prominent in medulla and cerebellum. Scale bar, 20 μm. (D) Immunostaining showed a punctate pattern of A_1R in the cortex of preterm infants at the corrected age of 26–27 weeks. Representative images of the cortex from 6 preterm brains are shown. Scale bar, 10 μm. (E) A_1R colocalized with a synaptic protein, PSD-95, in frozen sections of neonatal mouse brain (upper panel) and paraffin sections of preterm infant brains (lower panel). Scale bar, 20 μm.

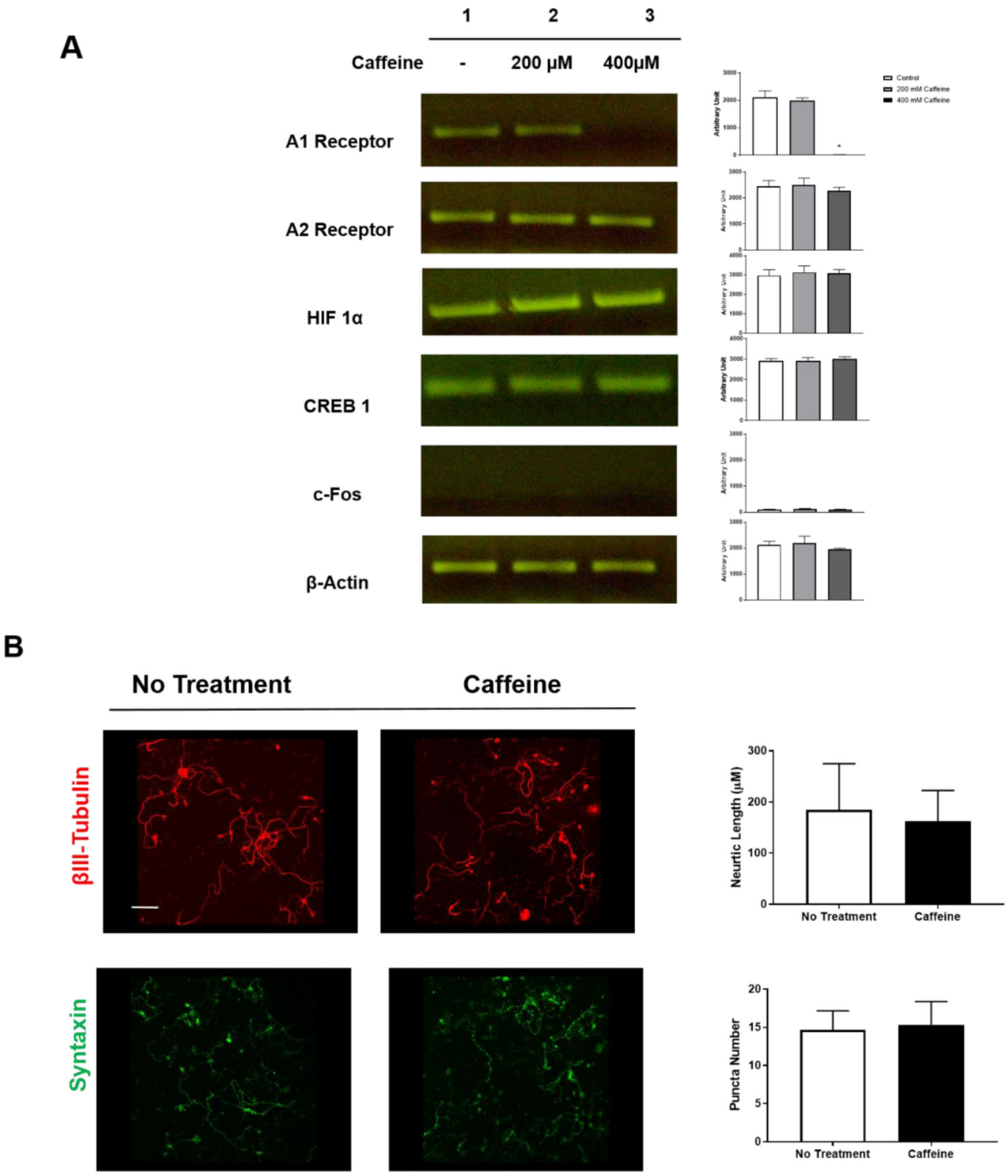
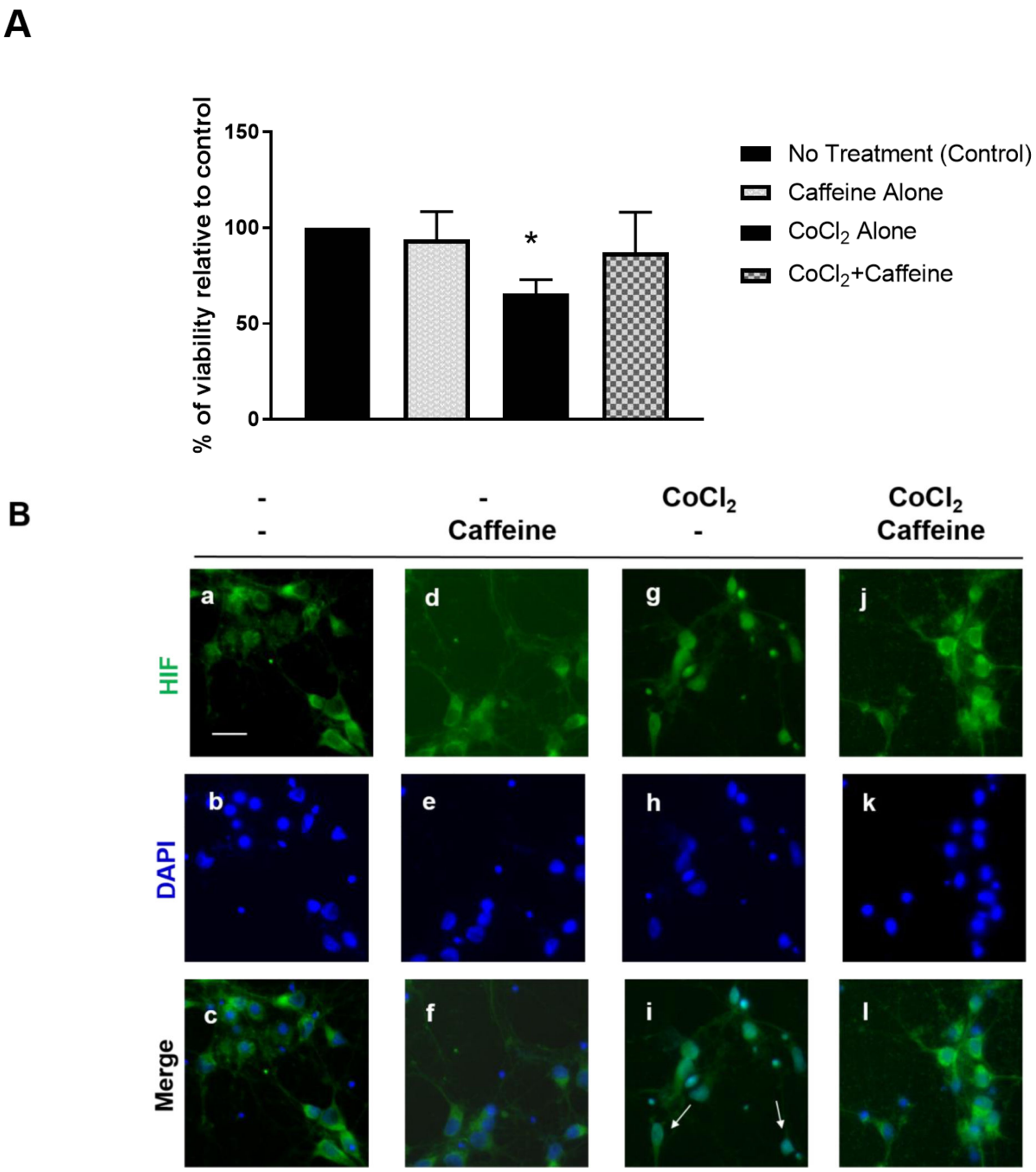


Figure 2.

(A) Treatment with 200 μM caffeine has minimal/no effect on the transcription of A_1R and $A_{2A}R$, HIF-1α, CREB-1 or c-Fos *in vitro*. Single cell RT/PCR was performed on neuronal culture that were treated with or without caffeine for 5 days (n=10). The intensities of the RT-PCR bands were quantified by densitometry in arbitrary units. The results of control and caffeine groups are as follows: A_1R (2104±138, 2000±136), $A_{2A}R$ (2446±130, 2500±150), HIF-1α (2978±17.5, 3141±19.3), CREB-1 (2928±64, 2933±88) and c-Fos (105±7.5, 132±7.6). The levels of β-actin were used as a loading control. The transcription levels of

CREB-1 and HIF-1α were discernible, but the level of c-Fos was barely detectable. A high dose of caffeine (400 μM) selectively decreased the transcription of A_1R (36±0.3, $p<0.001$) but not other tested factors. (B) Treatment with 200 μM caffeine did not affect neurite length (beta-III tubulin, red) (184.9±27.16 μm for control and 162.1±17.45 μm for caffeine treatment group) and synapse formation (syntaxin, green) (14.67±1.45 for control and 15.33±1.76 for caffeine treatment group). The quantitative results are shown on the right. Scale bar, 100 μm (n=5).



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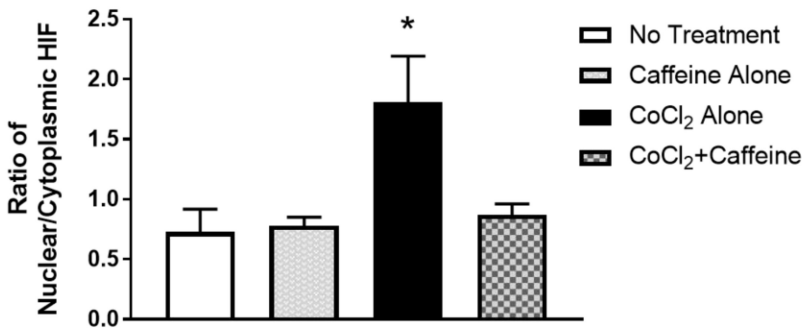
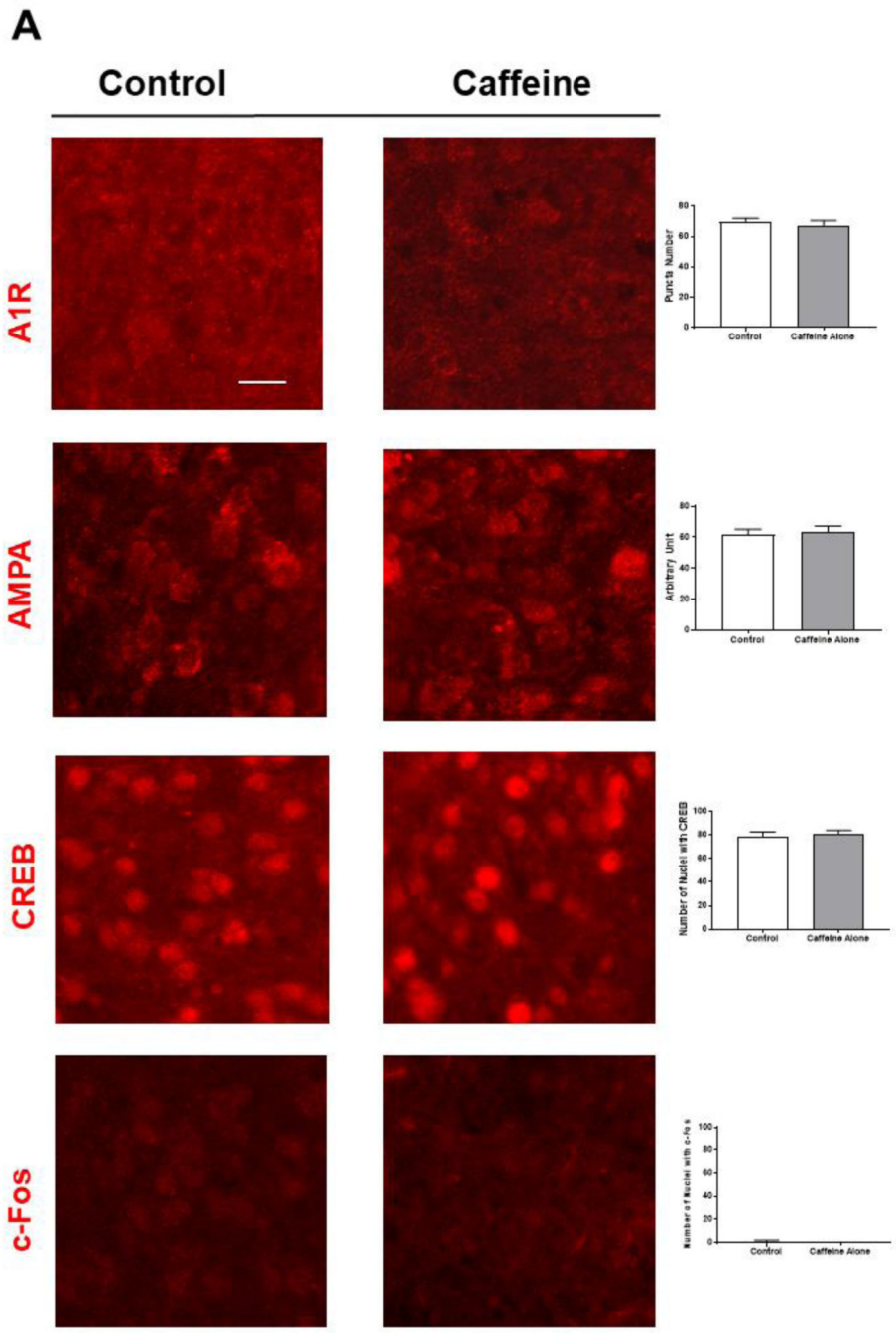


Figure 3.

Caffeine promoted cell survival after hypoxia and decreased $CoCl_2$-induced nuclear accumulation of HIF-1α *in vitro*. (A) Primary cortical neuronal cultures were treated with $CoCl_2$ or vehicle (control) for 24 h. Each group was divided into two subgroups that were either treated with 100 μM caffeine or vehicle for an additional 24 h. The viability of each treatment group was subsequently measured by MTT assays. Data are presented as percentage of viability in each group relative to that of the control group (control, 100%; caffeine alone, 94±7.1%; $CoCl_2$ treatment, 65.6±3.6%; $CoCl_2$+caffeine, 87.2±10.5% n=4. p<0.001).

(B) Treated neurons were double-immunostained to label HIF-1α (green, top row) and neuron-specific beta-III tubulin (red, not shown) and counter-stained with DAPI for nuclei (blue, middle row). The merged images of HIF-1α and DAPI are shown in the bottom row. Beta-III tubulin immunostaining (not shown) was used as a neuronal marker. In the absence of $CoCl_2$, HIF-1α was primarily localized in the cytoplasm regardless of whether neurons received caffeine or not (Lane 1, a, b, c and Lane 2, d, e, f). In contrast, $CoCl_2$ triggered nuclear co-localization of HIF-1α with DAPI (Lane 3, g.h.i), suggesting nuclear accumulation of HIF-1α. White arrows indicate co-localization of HIF-1α and DAPI. This nuclear accumulation of HIF-1α was alleviated by subsequent treatment with caffeine (Lane 4, j, k,l). Scale bar, 20 μm. (C) Quantification of the ratio of nuclear vs cytoplasmic HIF-1α in neurons of each treatment group (control, 0.73±0.07; caffeine alone, 0.78±0.03; $CoCl_2$ treatment, 1.81±0.17, $CoCl_2$ and caffeine, 0.82±0.04, p<0.001, n=5).



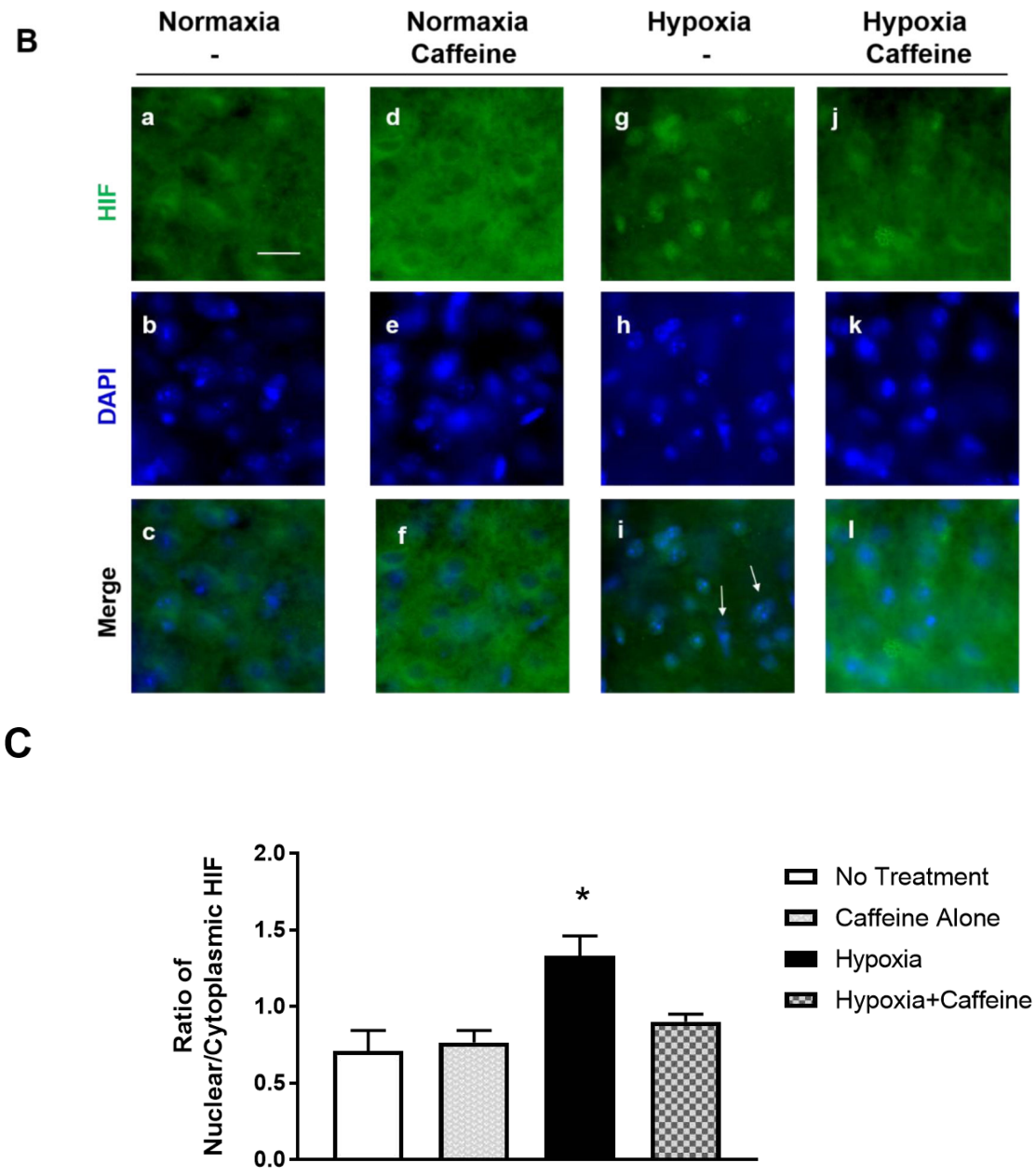


Figure 4.

Caffeine blocked hypoxia-induced nuclear accumulation of HIF1-α *in vivo*. (A) Immunostaining for A1R, AMPA receptors, CREB-1 and c-Fos showed that caffeine treatment has minimal/no effect on the expression of these factors in neonatal cortex (n=5). Scale bar, 20 μm. The quantitative results in control and caffeine-treated groups are shown on the right; the number of A_1R (70±1.15, 67.3±1.86) or AMPA receptor puncta (67.6±2, 63.6±2), the number of nuclear CREB-1 (79±2.1, 81.3±1.5) or c-Fos (1±0.58, 1±0.01). (B) Neonatal mouse pups were gavage fed with caffeine or vehicle from P4-P7. Subsequently, pups were placed under normoxic (room air) or hypoxic (8% O_2) conditions for 20 min prior to cardiac perfusion and isolation of their brains. Brain sections were immunostained with anti-HIF-1α (green, top row) and counter-stained with DAPI to show nuclei (blue, middle

row). The overlaid images of HIF-1α immunostaining and DAPI staining are shown in the bottom row. Representative cortical images are shown here. Under normoxic conditions, HIF-1α primarily distributed in the cytoplasm of cortical neurons whether mouse pups received caffeine therapy or not (Lane 1, a. b. c. and Lane 2, d, e, f). In pups without caffeine therapy, hypoxia induced nuclear accumulation of HIF-1α (Lane 3, g, h, i). White arrows indicate co-localization of HIF-1α and DAPI. Pre-treatment with caffeine prevented subsequent hypoxia-induced nuclear accumulation of HIF-1α in neonatal cortex (Lane 4, j, k, l). Scale bar, 20 μm. (C) Quantification of the ratio of nuclear vs cytoplasmic HIF-1α in neonatal cortex in each group (control, 0.71±0.05; caffeine alone, 0.77±0.03; hypoxia, 1.33±0.05; hypoxia and caffeine, 0.9±0.02, $p<0.001$, n=5)