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Caffeine regulates GABA transport via A_1R blockade and cAMP signaling

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ABSTRACT

Caffeine is the most consumed psychostimulant drug in the world, acting as a non-selective antagonist of adenosine receptors A_1R and $A_{2A}R$, which are widely expressed in retinal layers. We have previously shown that caffeine, when administered acutely, acts on A_1R to potentiate the NMDA receptor-induced GABA release. Now we asked if long-term caffeine exposure also modifies GABA uptake in the avian retina and which mechanisms are involved in this process. Chicken embryos aged E11 were injected with a single dose of caffeine (30 mg/kg) in the air chamber. Retinas were dissected on E15 for *ex vivo* neurochemical assays. Our results showed that [^{3}H]-GABA uptake was dependent on Na^+ and blocked at 4 °C or by NO-711 and caffeine. This decrease was observed after 60 min of [^{3}H]-GABA uptake assay at E15, which is accompanied by an increase in [^{3}H]-GABA release. Caffeine increased the protein levels of A_1R without altering ADORA1 mRNA and was devoid of effects on $A_{2A}R$ density or ADORA2A mRNA levels. The decrease of GABA uptake promoted by caffeine was reverted by A_1R activation with N6-cyclohexyl adenosine (CHA) but not by $A_{2A}R$ activation with CGS 21680. Caffeine exposure increased cAMP levels and GAT-1 protein levels, which was evenly expressed between E11-E15. As expected, we observed an increase of GABA containing amacrine cells and processes in the IPL, also, cAMP pathway blockage by H-89 decreased caffeine mediated [^{3}H]-GABA uptake. Our data support the idea that chronic injection of caffeine alters GABA transport via A_1R during retinal development and that the cAMP/PKA pathway plays an important role in the regulation of GAT-1 function.

1. Introduction (963 words)

Caffeine is the main psychostimulant drug consumed in the world (Heishman and Henningfield, 1992; Temple et al., 2017). Caffeine is a methylxanthine able to affect several behavioral, cognitive and physiological functions of the CNS, such as sleep, cognition, learning, memory or promoting neuroprotection (Ribeiro and Sebastiao, 2010). Indeed, caffeine (50 μM) selectively interferes on synaptic transmission and plasticity in the hippocampus through adenosine receptor blockade, in a way that A_1R mediates the impact of caffeine on synaptic transmission while $A_{2A}R$ regulates its impact on long term potentiation (LTP) (Lopes et al., 2019). However, doses higher than 0.5 mM are able to inhibit cyclic nucleotides phosphodiesterase enzymes, mobilize internal calcium stores and also block $GABA_A$ receptors (Ferre, 2008; Fredholm et al., 1999; Ribeiro and Sebastiao, 2010; Williams and Jarvis, 1988). The adenosine effects are mostly linked to the activation of its metabotropic receptors, which can be classified into four cloned subtypes, namely A_1, A_{2A}, A_{2B}, and A_3 (Ribeiro et al., 2002). A_1R and

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Abbreviation list

A1R	adenosine A1 receptor
A2AR	adenosine A2A receptor
ADORA1	adenosine A1 receptor
ADORA2A	adenosine A2A receptor
BSA	bovine serum albumin
Caf	caffeine
cAMP	cyclic adenosine 3″,5″-monophosphate
cDNA	Complementary DNA
CGS-21680	3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino] ethyl]phenyl]propanoic acid
CHA	(2R,3R,4S,5R)-2-[6-(cyclopentylamino)purin-9-yl]-5-(hydroxymethyl)oxolane-3,4-diol
CNS	central nervous system
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12
E	Embryonic age of days
ECL kit	Electrochemiluminescence kit
F	foward
GABA	γ-Aminobutyric acid
GATs	GABA transporters
H-89	N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride
HRP	horseradish peroxidase
INL	inner nuclear layer
IPL	inner plexiform layer
NO-711	1-[2-[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3 pyridinecarboxylic acid hydrochloride
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PVDF	Polyvinylidene fluoride
R	reverse
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
Ro 20-1724	4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one)
RPL27	60S ribosomal protein L27
TCA	trichloroacetic acid
TTBS	Tween 20 Tris-buffered saline

A_3R are coupled with a G_0/G_i protein, while $A_{2A}R$ and $A_{2B}R$ are coupled with a G_s protein (Pearson et al., 2003; Ribeiro et al., 2002; Sheth et al., 2014), all of those subtypes can act in favor of or against neurotransmission (Ribeiro and Sebastiao, 2010). Adenosine has many roles in the nervous system, such as neuroprotection, synapse development, and neurotransmission circuitry modulation (Dos Santos-Rodrigues et al., 2015; Ferreira and Paes-de-Carvalho, 2001; Fredholm, 2010; Paes-de-Carvalho et al., 2003; Stone, 2005; Wardas, 2002).

The retina is a well-established experimental model used in neurochemistry studies for more than 40 years, as reviewed by (Vergara and Canto-Soler, 2012), quoting "*Many of the major concepts in developmental biology, such as those of induction, competence, plasticity, and contact inhibition, are due to work done on the chick*", and many other pieces of information we have today came from this model, especially in the retinal field.

There are a plethora of studies that use this model showing its structure and the presence of several neurotransmitters. Retinal neurochemistry and cytoarchitecture can be modified by neurotransmitters release during development (Brito et al., 2012; Brito et al., 2016; Calaza Kda and Gardino, 2010; da Silva Sampaio et al., 2018; de Freitas et al., 2016; De Mello et al., 1976; de Melo Reis et al., 2008; De Sampaio Schitine et al., 2007; Dos Santos-Rodrigues et al., 2015; Ferreira et al., 2014; Freitas et al., 2016; Kubrusly et al., 2007; Kubrusly et al., 2018; Paes-De-Carvalho, 2002; Pereira et al., 2010; Pohl-Guimaraes et al., 2010). Therefore, signaling pathway interference by a drug, such as caffeine, could promote modifications in the normal development profile of the retina.

In the retina, the adenosinergic system regulates calcium influx, neuronal survival, photoreceptor coupling and neurotransmitter release (Hartwick et al., 2004; Li et al., 2013; Paes-de-Carvalho et al., 2003; Santos et al., 2000; Socodato et al., 2011). In fact, adenosinergic receptors are present in the retina of several species (Blazynski and Perez, 1991). The adenosine receptors activity was firstly described in the chick embryo retina by de Mello et al., in 1982, however, those receptors were characterized by immunocytochemistry and autoradiography five years later (Braas et al., 1987). Although A_1R is the main subtype expressed throughout the avian retina development, $A_{2A}R$ is observed just in specific ages and there is no evidence of $A_{2B}R$ nor A_3R expression in the avian retina (Dos Santos-Rodrigues et al., 2015). Recently, we have shown that caffeine is able to regulate GABA release in avian retina stimulated by aspartate via A_1R (Ferreira et al., 2014), initially demonstrated by (Rego et al., 2000). In addition, we described that caffeine modulates aspartate uptake in the rat retina via $A_{2A}R$ (de Freitas et al., 2016). GABA, the principal inhibitory neurotransmitter of the CNS, its synthetic enzyme and transporters are localized on horizontal and amacrine cells of the chick retina (Frederick, 1987; Hokoc et al., 1990). This amino acid is found as early as E6 in embryonic avian retinal tissue (Calaza Kda and Gardino, 2010; De Mello et al., 1976; Hokoc et al., 1990). GABA is preferentially released by neuronal GABA transporter type 1 (GAT-1), which is also responsible for 90% of GABA uptake (do Nascimento and de Mello, 1985; Ferreira et al., 1994; Melone et al., 2014). GABA uptake and release, in the chicken retina, is totally dependent on Na^+ and Cl^- ions presence (do Nascimento et al., 1998; do Nascimento et al., 2013; Scimemi, 2014), and its expression or activity is modulated by a wide array of neuronal pathways, such as protein kinases activity, second messengers (Bagley et al., 2005; Ferreira et al., 2014; Quick et al., 2004; Schitine et al., 2015) or by the presence of NO-711 blocker, a selective drug that interrupts GAT-1 function (Ferreira et al., 2014; Martins et al., 2018). The effects of adenosine receptors in the chicken retina during embryonic stages has been extensively described (Brito et al., 2012; Brito et al., 2016; Calaza Kda and Gardino, 2010; Dos Santos-Rodrigues et al., 2015; Ferreira et al., 2014; Paes-De-Carvalho, 2002; Pereira et al., 2010). Specifically, both A_1R and $A_{2A}R$ are already present and functional by E10, as discussed by (Brito et al., 2012).

Here, we investigated if a single injection of caffeine *in ovo* at the E11 stage is able to modify GABA uptake in the avian retina at E15. Our choice was based on a previous paper of our group that showed that a single acute dose of caffeine is able to modulate NMDA receptors at E15-E16 by A_1R blockade (Ferreira et al., 2014). We decided to block adenosinergic receptors from the onset of adenosine receptors until the end of retina synaptogenesis, in a period where synapses are being formed, evaluating the mechanisms involved in this process, as well as the adenosinergic receptors that are being modulated by caffeine during this embryonic stage.

2. Material and methods (1672 words)

2.1. Ethics statement

All procedures were approved by and in accordance with the protocol of Animal Care and Use Committee of Federal University of Rio de Janeiro 038/19 and are in conformity with Brazilian Law No. 11794/2008, the guidelines of the Brazilian Society for Neuroscience and

Behavior (SBNeC) and the Guide for the Care and Use of Laboratory Animals as endorsed by the National Institutes of Health.

2.2. Animals and drug treatment

Fertilized White Leghorn eggs (*Gallus gallus domesticus*) were staged according to (Hamburger and Hamilton, 1951), obtained from a local hatchery, stored in an incubator with controlled temperature and humidity. Caffeine was injected *in ovo* at embryonic day 11 (E11) in the air chamber of the eggs under sterile conditions (Fig. 1). Saline solution was used for the vehicle (control) group. The final estimated caffeine concentration inside of the egg is 30 mg/kg, as described (Brito et al., 2016). Briefly, non-toxic doses of caffeine (5–30 mg/kg of the egg) is able to selectively modulate adenosinergic receptors. Based on these results, we injected eggs with a single dose of 30 mg/kg caffeine, aiming to, in our experimental design, block only the adenosine receptor. After injection, the eggs were returned to the incubator, where they remained until the experimental day.

2.3. Retina dissection

On E15, vehicle or treated embryos were decapitated and the retinas were dissected in Ca^{2+}- and Mg^{2+}-free Hank's (CMF) solution. Then, the retinas were placed in p35 plastic dishes with cold Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) for the neurochemical assays. The number of animals utilized in the experiments was 120 White Leghorns embryos (240 retinas). There was no sorting by sex, all efforts were made to reduce the number of animals used and minimize animal suffering, utilizing alternatives in vivo techniques if available.

2.4. Materials

[^{3}H]-GABA specific activity 35 Ci/mmol was purchased from PerkinElmer (Massachusetts, USA). Caffeine (1,3,7-Trimethylpurine-2,6-dione), bovine serum albumin (BSA), Ro 20–1724 (4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one), NO-711 (1-[2-[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3 pyridinecarboxylic acid hydrochloride), CHA ((2R,3R,4S, 5R)-2-[6-(cyclopentylamino)purin-9-yl]-5-(hydroxymethyl)oxolane-3,4-diol), CGS-21680 (3-[4-[2-[[6-amino-9-[(2R,3R,4S, 5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid), H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), DAB (3.3-diaminobenzidine) and DMEM/F-12 were obtained from Sigma/RBI (St Louis, MO, USA). All other reagents were of analytical grade.

Caffeine (30 mg/kg) was injected *in ovo* at the air chamber; NO-711 (50 μM), CHA (100 nM), CGS 21680 (10 nM) and H-89 (10 μM) were utilized on *ex-vivo* explants of dissected retinal tissue 15 min (min) before the [^{3}H]-GABA uptake assay and H-89 was further utilized before tissue collection for Western Blot.

2.5. [^{3}H]-GABA uptake

Each retina was incubated for 1 hour (h) in 1 mL DMEM/F12 or in Tris-HCl solution pH 7.4 at 37 °C containing 0.5 μCi of [^{3}H]-GABA (35 Ci/mmol = 35.106 μCi) and 20 μM of non-radioactive GABA as a carrier. For specific groups, both vehicle and caffeine *ex vivo* explants were pre-incubated for 15 min in the presence of NO-711 (50 μM), CHA (100 nM), CGS 21680 (10 nM) or H-89 (10 μM), before [^{3}H]-GABA uptake assay. After 60 min of [^{3}H]-GABA incubation, the medium was totally removed and the tissue was washed out for three times with 3 mL cold Hank's (4 mM) solution (NaCl 128 mM; KCl 4 mM; $MgCl_2$ 1 mM; $CaCl_2$ 3 mM; Hepes 20 mM; Glucose 4 mM; pH = 7.4). This process was sufficient to wash out the radioactivity not taken up by the cells. Then, 1 mL of Milli-Q water (Millipore) was added to disrupt cell membranes, followed by a freeze-thaw cycle. Cell radioactivity was assayed using a liquid scintillation counter and normalized by protein concentration, estimated with (Lowry et al., 1951) protein assay.

2.6. [^{3}H]-GABA release

The retina was incubated for 1 h in 1 mL DMEM/F12 containing 0.5 μCi of [^{3}H]-GABA (35 Ci/mmol = 35.106 μCi) and 20 μM of non-radioactive GABA as a carrier. The medium was removed and the tissue was washed twice with 1 mL Hank's solution with glucose 4 mM (4 mM). Afterward, the retina was superfused with 0.5 mL of Hank's (4 mM) at 37 °C and incubated in an interval of 5 min. Cell radioactivity was assayed using a liquid scintillation counter and results were normalized to percentage of total in accordance with (Kubrusly et al., 1998).

2.7. Western Blot

Both retinas were extracted from the right and left eyes, pooled and homogenized with RIPA buffer containing a protease inhibitor cocktail to evaluate A_1R, $A_{2A}R$ and GAT-1 protein levels in the caffeine and vehicle groups. The concentration of protein was estimated using (Bradford, 1976) protein assay. Samples were diluted in buffer composed of 10% glycerol (v/v), 1% β-mercaptoethanol, 3% SDS, and 62.5 mM Tris base and boiled for 5 min. Approximately 45 μg of protein from each sample were electrophoresed in 10% SDS–PAGE and transferred to PVDF membranes (ECL-Hybond). Membranes were washed with Tween 20 Tris-buffered saline (TTBS) and blocked for 2 h with 5% non-fat milk 1% BSA in TTBS for A_1R and $A_{2A}R$ and 1% BSA in TTBS for GAT-1. After blockade, the membranes were incubated with the primary antibodies anti-A_1R (dilution of 1:200 in TTBS; Sigma-Aldrich), anti-$A_{2A}R$ (1:1000 in TTBS; Santa Cruz Biotechnology), anti-GAT-1 (1:500 in TTBS; Sigma-Aldrich), overnight at 4 °C. Membranes were rinsed in TTBS and incubated with anti-rabbit peroxidase-conjugated secondary antibody (1:5000 in TTBS; Sigma-Aldrich) for 2 h at room temperature. Following three washes using TTBS (10 min each), labeling was detected with an ECL kit (Amersham). Blots were re-probed with the anti-β-tubulin antibody (1:25000 in TTBS, Sigma-Aldrich) for 1 h at room temperature, rinsed in TTBS and incubated with anti-mouse

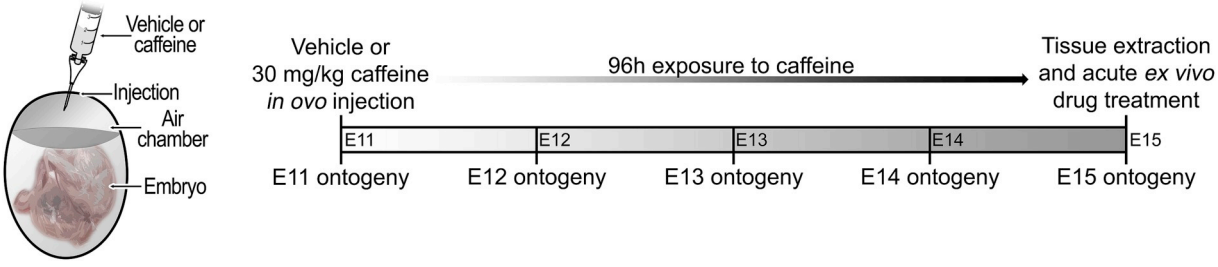

Fig. 1. Experiment design. (A) *In ovo* injection scheme. (B) Treatment and assays timeline.

peroxidase-conjugated secondary antibody (1:5000 in TTBS; Sigma-Aldrich) for 45 min at room temperature. Following another three TTBS washes. Immunolabeling was detected with the ECL kit. Band intensities were analyzed by using ImageLab 5.2.1 software (Bio-Rad Laboratories Inc).

2.8. Cyclic AMP assay

The retinal tissue was extracted and one retina per well was incubated in both vehicle and caffeine groups analyzed in DMEM/F12 with 500 μM of Ro 20–1724 for 60 min at 37 °C. The reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 10%. The cAMP was purified and assayed as described previously (de Mello et al., 1982). The protein was assessed using (Lowry et al., 1951) assay.

2.9. Real-time PCR

Total RNA was isolated from retinal tissue using TRIzol® Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Quality and purity were evaluated by measuring absorbance at 230, 260, and 280 nm cDNA was synthesized using Superscript III Kit (Invitrogen) from 1 μg of total RNA. Genes of interest were amplified with a StepOne Real-Time PCR System (Applied BioSystems) using the GoTaq® qPCR Master Mix (Promega, WI, USA) following cycle parameters: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min. Oligonucleotide primer sequences for ADORA1 (A_1R gene), ADORA2A (A_{2A}R gene) and the reference gene RPL27 (L27 gene) (Table 1) were designed using RealTime qPCR Assay Entry and synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, Iowa, EUA). Relative mRNA expression levels were calculated by $2^{-\Delta\Delta Ct}$ method. The expression of the L27 gene was stable for the experimental groups and the efficiency of each reaction was calculated using a serial dilution and varied from 95% to 105%. The data are expressed relative to the vehicle group, considered to be 1. The purity of the PCR products was assessed by melting curve analysis.

2.10. Immunohistochemistry

Retinas from E15 animals, exposed to caffeine or vehicle from E11 to E15, were dissected and immersed in 1 mL of Ringer solution (120 mM NaCl; 3 mM KCl; 30 mM $NaHCO_3$; 1 mM NaH_2PO_4; 1 mM $CaCl_2$; 1 mM $MgCl_2.6H_2O$; 10 mM glucose) perfused for the entire experiment with a gas mixture containing 95% O_2/5% CO_2 for 30 min. The pH of the Ringer was previously adjusted to 7.2–7.4. Then, retinas were fixed in 4% paraformaldehyde for 1 h, washed three times in 0.16 M phosphate buffer (pH = 7.2). To cryoprotect, retinas were incubated in a sucrose gradient (15 and 30%) and then frozen embedded in OCT in dry ice. The tissue was sectioned in a cryostat (12 μm) and retinal sections from both experimental groups (vehicle and caffeine) were collected on the same slide. This procedure guarantee that the immunohistochemistry procedure occurred simultaneously under the same conditions. Therefore, possible differences from experimental groups were not due to immunohistochemistry processing. Slides were maintained frozen until the day of the immunohistochemical procedure. Sections were incubated in blocking solution (2% BSA and 5% normal goat serum) for 1 h, and then with 1:3000 anti-GABA polyclonal antibody (Catalog #A2052, Sigma-Aldrich) overnight. In the following day, retinal sections were washed three times with PBS and then incubated in secondary antibody (1:200 goat-x-rabbit IgG) for 2 h. Finally, the avidin-biotin complex (1:50, Vector Labs) were applied to sections for 90 min. We use DAB as chromogen (0.05%), with 0.01% hydrogen peroxide for 10 min to reveal the immunolocalization. Then, sections were washed in phosphate-buffered saline ($CaCl_2$ 0.68 mM; KCl 2.7 mM; KH_2PO_4 1.47 mM; $MgSO_4$ 0.49 mM; NaCl 136 mM; Na_2HPO_4 8 mM; pH = 7.4) and mounted with buffered glycerol solution.

Digitized images were obtained in 40x magnification using the light microscope (DM2500, Leica Microsystems, Wetzlar, Germany). Quantification of the GABA immunoreactivity intensity used in the present paper has been used and previously validated (Maggesissi et al., 2009). Briefly, digitized images were converted to 8-bit grayscale in ImageJ and the intensity was quantified in the amacrine layer and inner plexiform layer using the freehand selection tool. The quantification was always obtained from at least three different fields per section and used at least three sections for each retinal treatment in each experiment.

2.11. Statistical analysis

For pairwise comparisons of independent measures, two-tailed Student's t-tests were used. Multiple comparisons as one-way or two-way ANOVAs followed by Bonferroni post hoc tests were when three or more groups were analyzed, we used. Data are expressed as mean ± standard error of the mean (SEM) and were obtained utilizing the software GraphPad Prism 6.01 (GraphPad Software, Inc.). For all tests, $p < 0.05$ was considered to be statistically significant.

3. Results (1036 words)

3.1. [^{3}H]-GABA uptake and release modulation by caffeine

In order to evaluate if a single injection of caffeine alters retinal GABA uptake levels at E15, embryonic eggs were treated with vehicle or caffeine 30 mg/kg at E11, under sterile condition, and performed [^{3}H]-GABA uptake assay four days later. Control retinas were incubated under different conditions: with Hank's (4 mM glucose) solution; with a modified Hank's (4 mM glucose) solution, replacing sodium ions for trizma salt; under low temperature (4 °C); or in the presence of 50 μM NO-711, a GAT-1 blocker. Caffeine-treated retinas were incubated with Hank's (4 mM glucose) solution or in the presence of 50 μM NO-711 (Fig. 2A). We added 20 μM of non-radioactive GABA as carrier to [^{3}H]-GABA uptake to improve the [^{3}H]-GABA uptake levels (Ferreira et al., 2014).

As shown, absence of Na^+ ions, or incubation at low temperature, or in the presence of 50 μM of NO-711 alone, or in addition to caffeine, completely blocked [^{3}H]-GABA uptake (Fig. 2A), while caffeine treatment alone reduced [^{3}H]-GABA uptake up to 41% of control levels (Control = 335.3 ± 28.2; n = 7; w/o Na^+ = 26.7 ± 2.8; n = 3; 4 °C = 20 ± 6.1; n = 3; Caffeine = 195.9 ± 19.9; NO-711 = 53.7 ± 3.7; n = 3; Caf + NO-711 = 84.3 ± 12.5 [^{3}H]-GABA uptake (fmol/mg/hour); n = 3).

As caffeine reduced [^{3}H]-GABA uptake, we then evaluated [^{3}H]-GABA release (Fig. 2B), since in the retina both are mediated by GAT-1. Caffeine increased [^{3}H]-GABA release by 65%, promoting a reversion of the transport. Addition of 50 μM of NO-711 prevented both basal and caffeine-mediated [^{3}H]-GABA release (Vehicle: Control = 1.80 ± 0.14; Caffeine = 2.97 ± 0.27; n = 8; NO-711: Control = 0.77 ± 0.17; Caffeine = 0.65 ± 0.15; [^{3}H]-GABA release (% of total); n = 4).

Table 1
Gallus gallus primer sequences used for qPCR assay.

Gene	Sequence
ADORA1	F 5′-AGCTGAAGATCGCCAAGT-3′ R 5′-GCAGGATGGGCAGAACAA-3′
ADORA2A	F 5′-CCCTCAGGTACAATGGCTTG-3′ R 5′-GACTTATTAGCACCCACCTCTTC-3′
RPL27	F 5′-TGTCGAGATGGGCAAGTTTATG-3′ R 5′-CGTCGATGTTCTTCACGATGAC-3′

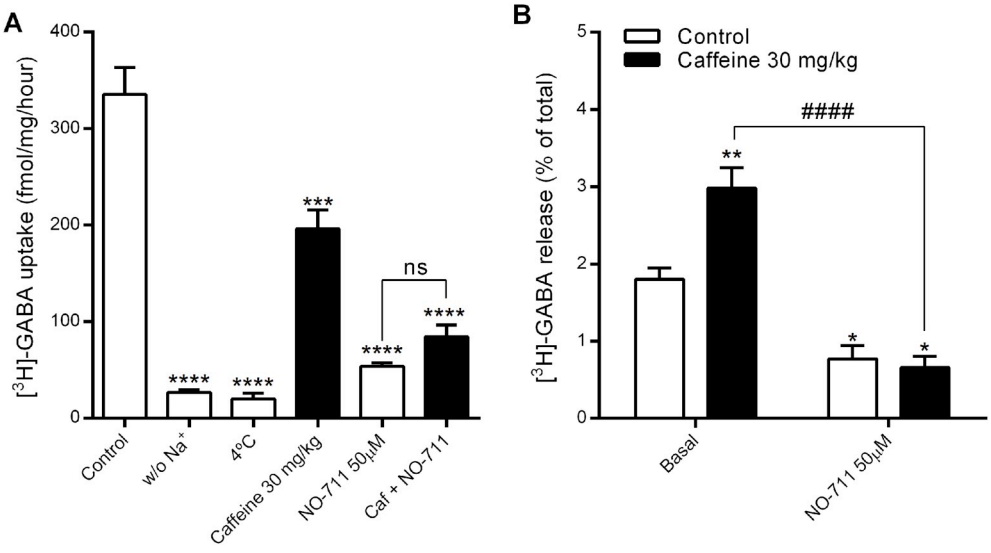

Fig. 2. Caffeine reduces [^{3}H]-GABA uptake via GAT-1 after 1h incubation. (A) [^{3}H]-GABA uptake is prevented by low temperature, replacement of sodium with TRIS in the incubation medium, GAT-1 inhibitor NO-711 50 µM and caf + NO-711, 96h after caffeine 30 mg/kg exposure, on E15 retinal tissue. (B) [^{3}H]-GABA release is increased 96h after caffeine 30 mg/kg exposure, on E15 retinal tissue and NO-711 50 µM acute exposure was able to inhibit both basal and caffeine-mediated increase of [^{3}H]-GABA release. Data are represented as mean ± S.E.M. *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001 from control and $^{\#\#\#\#}$p < 0.0001 from basal caffeine.

3.2. Caffeine increases A_1R, but not A_{2A}R protein density, without changing mRNA levels

A_1R and A_{2A}R total protein and mRNA expression levels were evaluated in E15 chick retinas exposed to vehicle or caffeine (Fig. 3A–E). The levels of A_1R (Fig. 3B) were higher after caffeine exposure, compared to vehicle (Control = 1.00 ± 0.16; n = 4; Caffeine = 2.05 ± 0.29; A_1R/Tubulin (% of control); n = 3). However, there was no increase of ADORA1 mRNA (Fig. 3C) (Control = 1.00 ± 0.02; Caffeine = 1.01 ± 0.06 mRNA expression of ADORA1 (relative to control); n = 5). Furthermore, there was no change in total protein levels of A_{2A}R (Fig. 3E) after caffeine exposure (Control = 1.00 ± 0.16; Caffeine = 0.85 ± 0.04; A_{2A}R/Tubulin (% of control); n = 3) nor in the expression of ADORA2A mRNA (Fig. 3F) (Control = 1.00 ± 0.02; Caffeine = 1.64 ± 0.43 mRNA expression of ADORA2A (relative to control); n = 5).

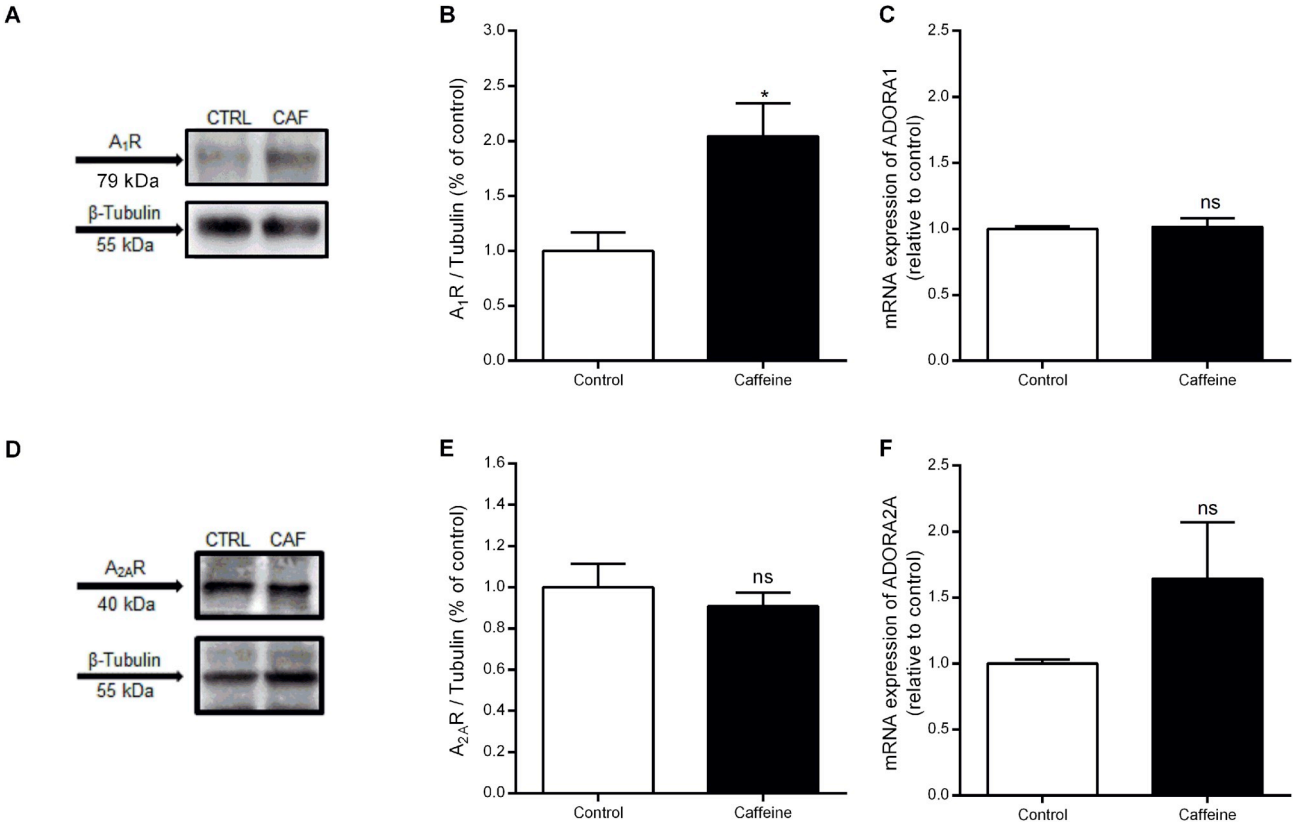

Fig. 3. Caffeine increases A_1R levels in a post-translational manner, without affecting A_{2A}R. (A) Western blotting of A_1R 79 kDa of vehicle and caffeine 30 mg/kg groups on E15 retina. (B) Densitometry analysis demonstrating an increase in A_1R levels on E15 after caffeine treatment. (C) Real-time PCR for the ADORA1 gene shows no modification in its expression after caffeine 30 mg/kg exposure, indicating a post-translational modification. (D) Western blotting of A_{2A}R on E15 of vehicle and caffeine 30 mg/kg groups. (E) Densitometry analysis shows no change in A_{2A}R protein levels on E15 after caffeine treatment. (F) Real-time PCR for the ADORA2A gene shows no modification in its expression after caffeine 30 mg/kg exposure. Control was normalized to 1 ± S.E.M and data are represented as % of control ± S.E.M. *p < 0.05, from control.

3.3. Decrease of [^{3}H]-GABA uptake induced by caffeine is mediated by A_1R

In order to test if activation of adenosine receptors could prevent the reduction of [^{3}H]-GABA uptake observed, selective receptor agonists were used, CHA 100 nM (A_1R agonist) or CGS 21680 10 nM ($A_{2A}R$ agonist), to determine which receptor was involved in the caffeine effect (Fig. 4A). We showed that A_1R agonist recovered the [^{3}H]-GABA uptake decrease promoted by caffeine when added 15 min before the GABA uptake assay. However, addition of the $A_{2A}R$ agonist alone, or combined to caffeine, had no effect compared to vehicle (Vehicle: Basal = 335.3 ± 28.2; n = 7; CHA 100 nM = 296.0 ± 19.0; n = 7; CGS 21680 10 nM = 278.7 ± 40.4; n = 3; Caffeine: Caffeine = 195.9 ± 19.9; n = 11; CHA 100 nM = 308.1 ± 36.0; n = 9; CGS 21680 10 nM = 195.0 ± 13.4 [^{3}H]-GABA uptake (fmol/mg/hour); n = 5).

3.4. Decrease of GABA uptake and increase of GABA release induced by caffeine involves GAT-1 and cAMP/PKA pathway modulation

Since GAT-1 is the major transporter for [^{3}H]-GABA uptake in the avian retina (Schitine et al., 2015), GAT-1 total protein levels were evaluated after vehicle injection between E11-E15 (Fig. 5A and B). As observed, there was no significant change of GAT-1 levels through retinal stages (E11 = 0.073 ± 0.004; E12 = 0.075 ± 0.013; E13 = 0.071 ± 0.004; E14 = 0.054 ± 0.002; E15 = 0.091 ± 0.003 GAT-1/Tubulin (a.u.); n = 4–3).

GAT-1, like other amino acid transporters, can be regulated by different signaling pathways (Ferreira et al., 2014). Indeed, as caffeine activates PKA function in rat retina cells (de Freitas et al., 2016), we investigated the effects of caffeine exposure for 96 h on the levels of GAT-1 (Fig. 5C and D). As shown, GAT-1 total protein levels were increased by caffeine (Control = 1.00 ± 0.14; n = 8; Caffeine = 1.53 ± 0.12 GAT-1/tubulin (% of control); n = 7). We also evaluated the GABA cell content in the chick retina exposed or not to caffeine by using an immunohistochemistry approach (Fig. 5E–H). We verified that caffeine exposure increased GABA cell content both in amacrine cells in the INL (Control = 1.00 ± 0.12; Caffeine = 1.28 ± 0.03 O.D. of GABA in amacrine cells; n = 3) and in processes in the IPL (Control = 1.00 ± 0.11; Caffeine = 1.44 ± 0.08 O.D. of GABA in IPL; n = 3).

Additionally, since adenosine receptors regulate the cAMP/PKA pathway, we also analyzed cAMP levels of retinal tissues exposed to vehicle or caffeine (Fig. 6A). As shown, caffeine exposure increased more than twofold the cAMP levels when compared to vehicle group on E15 retinal tissue (Control = 27.91 ± 4.01; Caffeine = 63.47 ± 7.43 cAMP levels (pmol/mg/hour); n = 3–4).

Corroborating with the findings that GAT-1 can be phosphorylated by PKA pathway, we also analyzed GAT-1 function by blocking PKA pathway with H-89 in retinas exposed to vehicle or caffeine during [^{3}H]-GABA uptake (Fig. 6B). As shown, PKA blockade with H-89 recovered the caffeine-induced decrease of [^{3}H]-GABA uptake, returning it to the basal levels (Vehicle: Control = 335.3 ± 28.2; n = 7; Caffeine = 195.9 ± 19.9; n = 11; H-89: Control = 438.3 ± 5.5; Caffeine = 446.9 ± 110.4 [^{3}H]-GABA uptake (fmol/mg/hour); n = 3).

4. Discussion (1109 words)

The adenosinergic receptors subtypes are highly expressed in the avian retina (Brito et al., 2016; Dos Santos-Rodrigues et al., 2015; Pearson et al., 2003; Ribeiro et al., 2002). According to (de Carvalho et al., 1992), A_1R is expressed around embryonic E10 in the avian retina, with a peak between E13-E16, while $A_{2A}R$ demonstrate a significant function around E14, increasing cAMP accumulation (de Mello et al., 1982). In our study, a single caffeine injection was performed on E11, when adenosine receptors are expressed and neurogenesis is virtually concluded (E12) (Mey and Thanos, 2000; Prada et al., 1991; Thanos and Mey, 2001). Our focus in this study is on the pharmacological effect of caffeine and its ability to block adenosine receptors A_1 and A_{2A} (Fredholm et al., 2005), modulating the GABAergic system. Both adenosinergic and GABAergic systems are present in the avian retina during the studied period (Calaza Kda and Gardino, 2010).

The adenosinergic system is involved with progenitor proliferation and cell cycle exit of several neurons in the vertebrate retina (Martins and Pearson, 2008). Adenosine modulates the vesicular release of neurotransmitters through their receptor activation (Paes-De-Carvalho, 2002; Sebastiao and Ribeiro, 2009).

Caffeine has a half-life higher than 100 h in human infants due to lower cytochrome P-450 activity and/or immaturity of acetylation and demethylation pathways (Fredholm et al., 1999). Furthermore, caffeine plasma levels are found at similar concentration levels in the CNS after

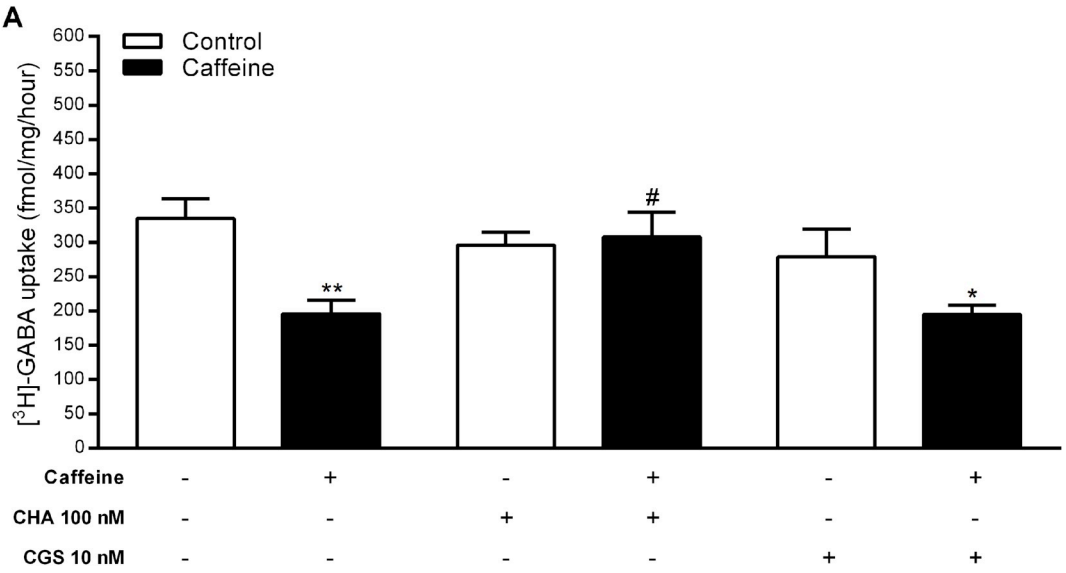

Fig. 4. A_1R, but not $A_{2A}R$ activation reverts GABA uptake decrease induced by caffeine. (A) Addition of 100 nM CHA in *ex vivo* retinal explants, 15 min prior to uptake assay, reverts caffeine 30 mg/kg [^{3}H]-GABA uptake reduction to basal values, while acute treatment with 10 nM CGS 21680 had no effect; both drugs did not affect uptake levels without caffeine when compared to basal. Data are represented as mean ± S.E.M. *p < 0.05, **p < 0.01 from basal control; #p < 0,05 from basal caffeine.

1 h of consumption (Nehlig et al., 1992). In our model, caffeine injection in the egg allows the study of embryonic development without teratogenic effects, including the incorrect development of the eye (Ma et al., 2012, 2014). Even though we followed the same injection protocol published by (Brito et al., 2016), the exact concentration of caffeine that reaches the retina, in our study, is not known. However, we did not observe any macroscopic malformation nor embryonic death induced by caffeine injection, although we have observed a strong effect on adenosinergic A1R and pathway activation, indicating that,

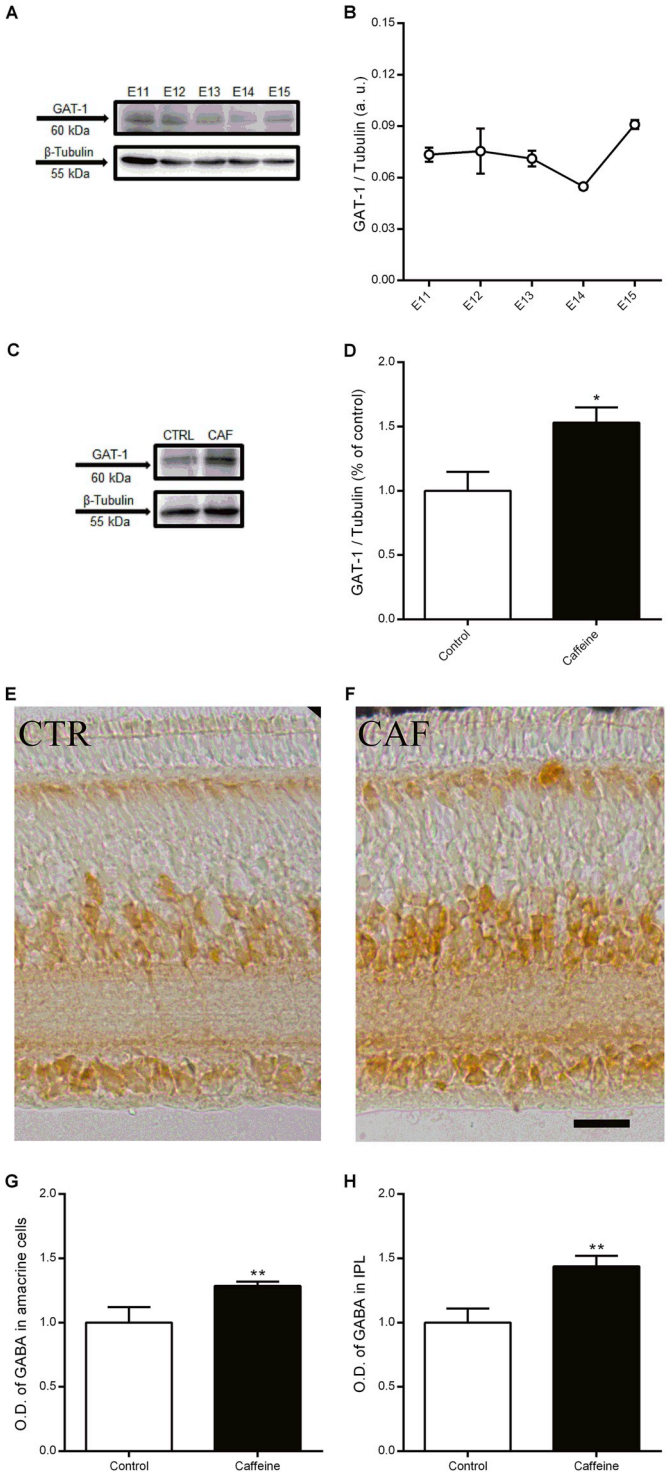

Fig. 5. GAT-1 levels during the development and caffeine increase in GAT-1 levels and GABA-positive amacrine cells and IPL processes. (A) Western blotting to GAT-1 on E11, E12, E13, E14, E15. (B) Densitometry analysis demonstrating no change in GAT-1 total protein levels of chick embryos retina aged E11-E15. (C) Western blotting of GAT-1 on E15 of vehicle and caffeine 30 mg/kg groups. (D) Densitometry analysis of GAT-1 protein levels in chick retina indicating an increase of the protein levels after 96h caffeine 30 mg/kg exposure *in ovo*. (E) Photomicrographs of radial sections of E15 retinas processed immunohistochemically for GABA from vehicle injected embryo. (F) Photomicrographs of radial sections of E15 retinas processed immunohistochemically for GABA from caffeine 30 mg/kg injected embryo. (G) Optical densitometry analysis of GABA-immunoreactivity in amacrine cells from retinas exposed to caffeine 30 mg/kg in relation to control. (H) Optical densitometry analysis of GABA-immunoreactivity in the IPL from retinas exposed to caffeine 30 mg/kg in relation to control. Data are represented as mean ± S.E.M or the control was normalized to 1 ± S.E.M and data are represented as % of control ± S.E.M. *p < 0.05; **p < 0.01 from basal control.

caffeine injection, in the air chamber, is reaching the chicken retina embryo. In addition, it is important to mention that caffeine alters the organization of the GABAergic system in mammals (Silva et al., 2013).

Measurements using HPLC describe elevated caffeine level in the chick embryo brain after a single injection of caffeine in the air sac of the egg, possibly reaching the embryonic brain (Li et al., 2012). As discussed (Brito et al., 2016), and in accordance with (Reagan-Shaw et al., 2008), the appropriate normalizations to extrapolate animal dose to human depends on body surface area, the 30 mg/kg dose used in our experimental design would parallel a human equivalent dose of 3.64 mg/kg, which is close to the daily dosage intake of caffeine consumed by humans (2.4–4.0 mg/kg) (Fredholm et al., 1999).

GABA is the major inhibitory neurotransmitter the CNS (Watanabe et al., 2002). In the avian retina, GABA is mostly expressed in the horizontal and amacrine neurons (Calaza Kda and Gardino, 2010), and also in Müller glial cells in retinal cell cultures (Schitine et al., 2015). As observed in our results, removal of Na^+ or addition of No-711 (50 μM) blocked [^{3}H]-GABA uptake, reinforcing the prevalence of GAT-1 on GABA uptake in the chick retina. These results are in accordance with data reported in the mouse frontal cortex with a high protein levels of GAT-1 (Martins et al., 2018).

Our previous data demonstrated that caffeine, in acute doses, alters protein levels and function of EAATs in the rat retinal tissue (de Freitas et al., 2016) and potentialize GABA release induced by aspartate in the avian retinal cell culture (Ferreira et al., 2014). These effects were shown to be mediated by $A_{2A}R$ and A_1R blockade, respectively, and suggest that caffeine exposure is able to modify extracellular GABA homeostasis in the developing retina in order to evoke neuroprotection.

Caffeine treatment significantly reduced [^{3}H]-GABA uptake after 60 min, mediated by GAT-1, which activity was reduced, as previously observed by (Ferreira et al., 2014). Selective A_1R protein levels were also increased, induced by caffeine treatment. However, A_1R level increase was not followed by an ADORA1 mRNA expression rise, indicating a post-translational effect of caffeine upon the A_1R, as described previously in the rat brain (Johansson et al., 1993), or through selective A_1R antagonist in the avian retina (Brito et al., 2012). We also looked into the $A_{2A}R$ total protein levels and ADORA2A mRNA, both of which were not modified by caffeine treatment. A_1R is mostly coupled to G_i protein and rarely linked to PKC pathway (Cristovao-Ferreira et al., 2013), suggesting that caffeine blocks A_1R receptors, leading to an increment of cAMP levels and PKA pathway activation. In fact, data demonstrate that adenosine receptors can be highly expressed after cAMP analogs stimuli, which regulate translation of A_1R, as observed in cell culture of the avian retina (Pereira et al., 2010).

The increase in A_1R levels might play a role in neuroprotection since it counteracts glutamate release, inducing neurons to hyperpolarize (Cunha, 2016). The action of adenosine, activating A_1R and breaking

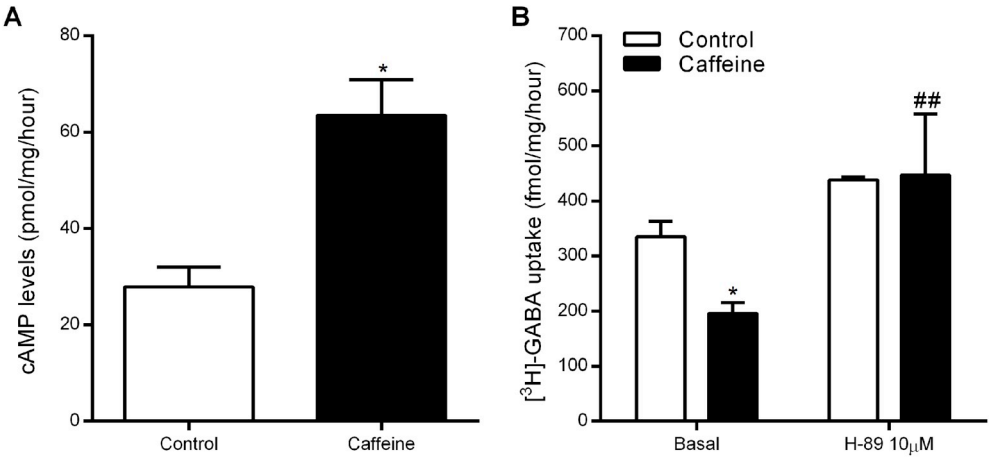

Fig. 6. The involvement of cAMP/PKA pathway on GABA uptake modulation by caffeine. (A) cAMP levels are increased by 227% when compared to basal levels after caffeine 30 mg/kg treatment. (B) Acute exposure, 15 min prior to uptake assay, to 10 μM H-89 in *ex vivo* retinal explants was also able to revert the [^{3}H]-GABA uptake reduction promoted by caffeine 30 mg/kg exposure, further pointing to cAMP/PKA pathway. Data are represented as mean ± S.E.M. *p < 0.05 from basal control; $^{\#\#}$p < 0,01 from basal caffeine.

excitatory transmissions, seems to be clear. However, the observed neuroprotection by adenosine receptors activation is due to glial communication, neurogenesis, and/or growth factors release (Gomes et al., 2011). In addition to these mechanisms, the literature has demonstrated that cAMP/PKA pathway activation also promotes a neuroprotective effect via adenosine in cultured chicken retina in the developmental stage studied (Socodato et al., 2011). Corroborating the hypothesis that increased A_1R levels regulate neuroprotection against excitatory cellular insults, our results demonstrated that A_1R activation, through the actions of CHA, but not of CGS 21680, is able to prevent caffeine effects on GABA uptake levels. Nonetheless, the increase in GABA-positive amacrine cells and IPL processes, as well as the increase of GABA release promoted by caffeine, might indicate a possible increase in the neuroprotection against excitotoxicity.

As blockade of A_1R by caffeine increases intracellular cAMP levels, phosphorylates GAT-1 and decreases [^{3}H]-GABA uptake while increasing its release, GAT-1 could be a potential target to regulate cellular excitability that is interrupted by drug abuse. In light of this and in agreement with our previous work (Ferreira et al., 2014), it is possible that during CNS development, caffeine influences the formation, function, and strength of synapses. Our findings show that a single injection of caffeine at E11 was able to modify receptors protein levels and function as well as GABAergic transport at E15. While we cannot ascertain to what extent caffeine is metabolized, the main point in our study is that a single dose of caffeine during the onset of adenosinergic receptors protein levels induces a change in the homeostasis of GABA, the main inhibitory neurotransmitter, regardless of its metabolization.

5. Conclusions

Our main conclusions are i) [^{3}H]-GABA uptake is mediated by GAT-1, which is expressed throughout the embryonic stage evaluated from E11 and constantly maintained its protein levels until E15 in the avian retina; ii) At this developmental period, caffeine decreases [^{3}H]-GABA uptake while increasing its release both via GAT-1 action; iii) Caffeine exposure increases GAT-1 and A_1R protein levels, whilst having no effect on $A_{2A}R$ protein level nor on A_1R or $A_{2A}R$ mRNA levels; iv) Caffeine treatment increases intracellular cAMP levels as well as GABA-positive amacrine cell and IPL processes; v) [^{3}H]-GABA uptake decrease is reversed by CHA and H-89, but not by CGS 21680; vi) PKA blocker prevents [^{3}H]-GABA uptake induced by caffeine.

It is worth noting that caffeine alters GABA transport after a 96 h period of exposure at embryonic day 15. Since this work is focused on the developmental effects during the embryonic stages, we do not possess any post-hatching data.

In conclusion, blockade of the adenosinergic system, particularly A_1R during retinal development, modulates GABA homeostasis, which seems to be dependent on cAMP signaling (Fig. 7).

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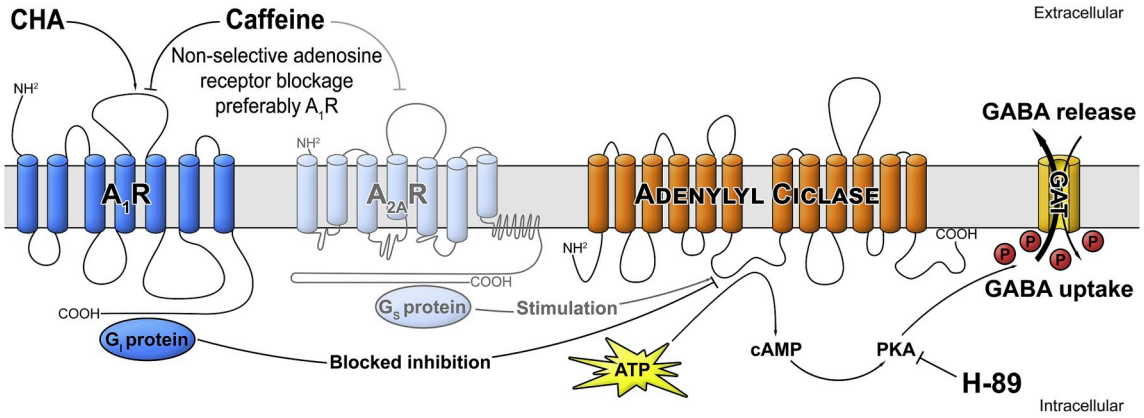

Fig. 7. Caffeine/A_1R/cAMP/GAT-1 interaction. Schematic model indicating the effect of caffeine on GABA transport regulation on avian retina cells. The blockade of A_1R leads to an increase of cAMP/PKA signaling reducing GABA uptake and increasing its release. A_1R, a G-coupled protein associated with a G_i is blocked by caffeine, thus promoting an increase of cAMP/PKA pathway, increasing intracellular cAMP levels. This change in cAMP levels promotes an increase of PKA and GAT-1 phosphorylation by PKA. As a consequence, GAT-1 has its transporter activity modified, decreasing GABA uptake and increasing GABA release. Acute activation of A_1R receptor by CHA is able to revert caffeine effect, returning GABA uptake to basal levels, as well as the blockade of the PKA pathway by H-89 treatment. Furthermore, $A_{2A}R$ appears to not participate in caffeine action.

agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

None.

Acknowledgments/conflict of interest disclosure

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