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ORIGINAL ARTICLE

Adenosine A_{2A} and histamine H_3 receptors interact at the cAMP/PKA pathway to modulate depolarization-evoked [^{3}H]-GABA release from rat striato-pallidal terminals

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Abstract

We previously reported that the activation of histamine H_3 receptors (H_3Rs) selectively counteracts the facilitatory action of adenosine A_{2A} receptors (A_{2A}Rs) on GABA release from rat globus pallidus (GP) isolated nerve terminals (synaptosomes). In this work, we examined the mechanisms likely to underlie this functional interaction. Three possibilities were explored: (a) changes in receptor affinity for agonists induced by physical A_{2A}R/H_3R interaction, (b) opposite actions of A_{2A}Rs and H_3Rs on depolarization-induced Ca^{2+} entry, and (c) an A_{2A}R/H_3R interaction at the level of adenosine 3′,5′-cyclic monophosphate (cAMP) formation. In GP synaptosomal membranes, H_3R activation with immepip reduced A_{2A}R affinity for the agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate (CGS-21680) (K_i control 4.53 nM; + immepip 9.32 nM), whereas A_{2A}R activation increased H_3R affinity for immepip (K_i control 0.63 nM; + CGS-21680 0.26 nM). Neither A_{2A}R activation nor H_3R stimulation modified calcium entry through voltage-gated calcium channels in GP synaptosomes, as evaluated by microfluorometry. A_{2A}R-mediated facilitation of depolarization-evoked [2,3-^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release from GP synaptosomes (130.4 ± 3.6% of control values) was prevented by the PKA inhibitor H-89 and mimicked by the adenylyl cyclase activator forskolin or by 8-Bromo-cAMP, a membrane permeant cAMP analogue (169.5 ± 17.3 and 149.5 ± 14.5% of controls). H_3R activation failed to reduce the facilitation of [^{3}H]-GABA release induced by 8-Bromo-cAMP. In GP slices, A_{2A}R activation stimulated cAMP accumulation (290% of basal) and this effect was reduced (− 75%) by H_3R activation. These results indicate that in striato-pallidal nerve terminals, A_{2A}Rs and H_3Rs interact at the level of cAMP formation to modulate PKA activity and thus GABA release.

Keywords Adenosine A_{2A} receptor · Histamine · Histamine H_3 receptor · Globus pallidus · Basal ganglia · GABA release

Abbreviations

A_{2A}R	Adenosine A_{2A} receptor
GABA	γ-Aminobutyric acid
GP	Globus pallidus
H_3R	Histamine H_3 receptor

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Introduction

Adenosine is an important modulator of the function of the mammalian central nervous system (CNS) [1]. Adenosine is produced by the ectoenzymatic breakdown of ATP co-released with several classical neurotransmitters and neuromodulators, such as acetylcholine, noradrenaline, γ-amino butyric acid (GABA), glutamate, and dopamine [2]. In addition, neurons and astrocytes can directly release adenosine formed intracellularly via nucleoside transporters [3]. Four G protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3) mediate the actions of adenosine in the CNS [4], and high expression of A_{2A} receptors (A_{2A}Rs) is found in the striatum, nucleus accumbens, globus pallidus (GP), and olfactory tubercle, with low expression levels elsewhere in the brain [1].

The GP belongs to the basal ganglia, a group of subcortical neuronal nuclei involved in the control of motor

behavior, among other functions [5]. The GP has therefore been implicated in the pathophysiology of motor disorders, for example Parkinson's disease, in which alterations in the pattern and synchrony of discharge of pallidal neurons have been reported [6]. The main synaptic input to the GP is provided by a sub-population of GABAergic striatal neurons that preferentially express dopamine D_2 receptors and enkephalins [5]. These neurons also express high levels of A_{2A}Rs [7], coupled to $G\alpha_s$ proteins and thus to the adenosine 3′,5′-cyclic monophosphate (cAMP)/PKA pathway [8], and whose activation facilitates GABA release in rat GP [9–13].

The GP is innervated by histaminergic fibers [14] and striato-pallidal neurons express a high density of histamine H_3 receptors (H_3Rs) both on their bodies [15] and nerve terminals [16]. In a previous study, we showed that the activation of H_3Rs, coupled to $G\alpha_{i/o}$ proteins, selectively counteracted the facilitatory action of A_{2A}R stimulation on depolarization-evoked [2,3-^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) release from rat GP isolated nerve terminals (synaptosomes) [16], and in this work, we have examined the mechanisms likely to underlie the functional interaction reported. Three possibilities were addressed: (a) the modulation by H_3R activation of A_{2A}R affinity for agonists, due to a physical A_{2A}R/H_3R interaction; (b) opposite actions of A_{2A}Rs and H_3Rs on depolarization-induced Ca^{2+} entry through voltage-activated channels; and (c) a functional A_{2A}R/H_3R interaction at the level of cAMP formation.

A preliminary account of this work was presented in the abstract form to the European Histamine Research Society [17].

Methods

Animals

Rats (males, Wistar strain, 250–300 g), bred in the Cinvestav facilities, were used in the experiments. All procedures were approved by the Cinvestav Animal Care Committee and followed the guidelines for the care and use of laboratory animals issued by the National Institutes of Health (NIH Publications No. 8023, revised 1978) and the Mexican Council for Animal Care (NOM-062-ZOO-1999). All efforts were made to minimize animal suffering and to use only as many animals were required for proper statistical analysis.

Preparation of slices and synaptosomes

Animals were decapitated, the brain was quickly removed from the skull, and the forebrain was cut and immersed in ice-cold Krebs-Henseleit solution. Coronal slices (300 μm thick) were then obtained with a vibratome (World Precision Instruments, Sarasota, FL), and the pallidal tissue was carefully dissected from the slices [16]. The composition of the Krebs-Henseleit solution was (mM) NaCl 116, KCl 3, $MgSO_4$ 1, KH_2PO_4 1.2, $NaHCO_3$ 25, and D-glucose 11 (pH 7.4 after saturation with O_2/CO_2, 95:5% *v*/*v*). This solution did not contain $CaCl_2$ in order to reduce excitotoxicity. Synaptosomes were prepared from GP slices (seven rats per experiment) as described previously [16].

Radioligand binding to rat GP synaptosomal membranes

The synaptosomal pellet was resuspended in 20 ml of a hypotonic solution (10 mM Tris-HCl, 1 mM EGTA, pH 7.4, 4 °C). After 20 min at 4 °C, the suspension was centrifuged (32,000×*g*, 20 min, 4 °C) and the pellet (synaptosomal membranes) was resuspended in incubation buffer (50 mM Tris-HCl, 5 mM $MgCl_2$ pH 7.4). Protein contents were determined by the bicinchoninic acid assay (BCA; Pierce, Rockford, IL), using bovine serum albumin (BSA) as standard.

Binding of *N*-α-[methyl-^{3}H]-histamine ([^{3}H]-NMHA) to H_3Rs present in synaptosomal membranes (~ 50 μg protein aliquots) was performed as described in detail elsewhere [18]. For [^{3}H]-CGS-21680 binding to A_{2A}Rs, saturation analysis was performed in 100 μl buffer containing [^{3}H]-CGS-21680 (0.01–12 nM) and ~ 50 μg protein. Nonspecific binding was determined as that insensitive to the A_{2A}R antagonist ZM-241385 (10 μM). For inhibition experiments, membranes were pre-incubated (15 min, 30 °C) with adenosine deaminase (2 U/ml) to eliminate the endogenous adenosine and then incubated with [^{3}H]-CGS-21680 (~ 4 nM) and increasing concentrations (10^{-10}–10^{-5} M) of unlabelled 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate (CGS-21680). After 2 h at 25 °C, incubations were terminated by filtration through Whatman GF/B glass fiber paper, pre-soaked in 0.3% polyethyleneimine. Filters were soaked in 3-ml scintillator and the tritium content was determined by scintillation counting.

Saturation binding data were fitted to a hyperbola by non-linear regression with GraphPad Prism 5 (Graph Pad Software, San Diego, CA). Inhibition curves were fitted to a logistic (Hill) equation and values for inhibition constants (K_i) were calculated according to the equation [19]: $K_i = IC_{50} / 1 + \{[D] / K_d$, where $[D]$ is the concentration of radioligand present in the assay and K_d the mean value for the equilibrium dissociation constant estimated from the corresponding saturation analysis.

Depolarization-evoked [^{3}H]-GABA release from GP synaptosomes

Experiments were performed as described in detail elsewhere [16]. Briefly, synaptosomes were suspended in Krebs-Ringer-Hepes solution supplemented with 10 μM aminooxyacetic acid, 2 U/ml adenosine deaminase, and a mixture of [^{3}H]-

GABA/GABA (80 nM/3 μM). After incubation for 30 min at 37 °C, the synaptosomal suspension was apportioned randomly between the chambers of a superfusion apparatus (15 chambers in parallel; 100 μl per chamber) and perfused (1 ml/min) with Krebs-Ringer-Hepes solution. The composition of this solution was (mM) NaCl 113, $NaHCO_3$ 25, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.8, D-glucose 15, and Hepes 20, at pH 7.4 with NaOH.

Synaptosomes were perfused for 20 min before the collection of 17 fractions of 1 ml (1 min) each. [^{3}H]-GABA release was stimulated by switching to a Krebs-Ringer-Hepes solution containing high K^+ (20 mM, KCl substituted for NaCl) for fractions 4 and 13, returning to normal solution between these fractions and after the second K^+ stimulus. Drugs under test were present 5 min (CGS-21680, 8-Br-cAMP, and forskolin) or 8 min (immepip and H-89) before and throughout the second K^+ stimulus (i.e., fractions 8–13 for CGS-21680, 8-Br-cAMP and forskolin, and fractions 5–13 for immepip and H-89). The double-pulse protocol allows for the same synaptosomal sample being the control for the effect of drugs under test.

To allow for variations between chambers, fractional values were transformed to a percentage of the first fraction. To test for statistical differences between treatments, after subtraction of basal release, the area under the release curve for six fractions after the change to high K^+ (i.e., fractions 3–8 and 12–17) was determined for each individual chamber and the ratio of the second over the first K^+ stimuli (S2/S1) was calculated.

cAMP accumulation assay

GP punches (2 mm diameter) were obtained from brain coronal slices (300 μm thick) in Krebs-Henseleit solution with no $CaCl_2$ added. After equilibrium for 30 min at 37 °C in the same solution containing 1.8 mM $CaCl_2$, punches were placed in plastic tubes (two per tube) and incubated (15 min, 37 °C) in 200 μl Krebs-Henseleit solution containing adenosine deaminase (0.5 U/ml) and 1 mM 3-isobutyl-1-methylxantine (IBMX). Drugs under test were added in a 10-μl volume and incubations were continued for 30 min. Reactions were stopped by adding 1 ml of ice-cold Krebs-Henseleit solution, tubes were placed on ice, and the solution was aspirated before adding 25 μl of ice-cold HCl (1 M). After 30 min at 4 °C, samples were neutralized (25 μl 1 M NaOH and 100 μl 1 M Tris-HCl pH 7.0) and centrifuged (15,000×*g*, 3 min, 4 °C).

Endogenous cAMP was determined by a competition assay [20]. Briefly, samples of the extracts (50 μl) were mixed with 75 μl incubation buffer containing a crude supernatant from bovine adrenal medulla and [2,8-^{3}H]-adenosine 3′,5′-cyclic phosphate ([^{3}H]-cAMP; 10 nM). After 2.5 h at 4 °C, incubations were terminated by filtration over GF/B filters presoaked in 0.3% polyethylenimine followed by three washes with 1-ml ice-cold deionized water. Retained radioactivity was determined by liquid scintillation, and the amount of cAMP present in each sample was calculated by extrapolation to a standard cAMP curve (10^{-12}–10^{-5} M). The composition of the incubation buffer was 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, and 5 mg/ml BSA, pH 7.0 at 4 °C.

Microfluorometry

Synaptosomes were resuspended in Krebs-Ringer-Hepes solution containing the fluorescent Ca^{2+} indicator dye Fura 2-AM (1 μM) and 3% BSA and plated on recording plastic chambers previously coated with concanavalin A (2 mg/ml). After 60 min at 37 °C in the dark, the synaptosomes were rinsed with Krebs-Ringer-Hepes solution. The recording chamber was positioned on a TMD inverted microscope (Nikon, Japan) coupled to an RF-F3010 microfluorometer (Photon Technology International, South Brunswick, NJ). Changes in the $[Ca^{2+}]_i$ were determined by measuring the fluorescence ratio (510 nm) after excitation with lights of either 340 or 380 nm wavelengths. Fura 2 recordings were acquired at a frequency of 20 Hz and the background fluorescence at 340 and 380 nm was determined from synaptosome-free areas of the chamber.

Synaptosomes were perfused with Krebs-Ringer-Hepes solution at a rate of 1 ml/min. For two-pulse experiments, drugs or KCl (25 mM, substituted for NaCl) were applied in the perfusion solution. To test for drug effects, after subtraction of basal fluorescence, the area under the curve for the K^+-induced increase in fluorescence was determined and the ratio of the second over the first K^+ stimuli (S2/S1) was calculated.

Statistical analysis

Data are presented are means ± standard error (SEM), unless otherwise indicated. Statistical comparisons were performed with Student's *t* test or one-way ANOVA and post hoc Dunnett's or Tukey's tests (GraphPad Prism 5.0) as appropriate. Statistical significance was set at $P \leq 0.05$.

Drugs

The following drugs were purchased from Sigma-Aldrich (St. Louis, MO): adenosine deaminase (from bovine spleen), aminooxyacetic acid hemihydrochloride, cAMP, CGS-21680, histamine dihydrochloride, IBMX, and immepip dihydrobromide. Fura 2-AM was from Molecular Probes (Thermo Fisher Scientific, Waltham, MA). [^{3}H]-GABA (82 Ci/mmol), *N*-α-[methyl-^{3}H]-histamine (83.4 Ci/mmol), [^{3}H]-cAMP (34 Ci/mmol), and [^{3}H]-CGS-21680 (35.2 Ci/mmol) were from PerkinElmer (Boston, MA). For the cAMP accumulation assay, a crude supernatant from bovine adrenal medulla was used.

Results

[3H]-CGS-21680 binding to GP synaptosomal membranes

Saturation binding of the $A_{2A}R$ agonist [^{3}H]-CGS-21680 [21] to membranes from GP synaptosomes (Fig. 1a) yielded maximum specific binding (B_{max}) 454 ± 77 fmol/mg protein (mean ± SEM, five experiments) and equilibrium dissociation constant (K_d) 3.98 nM (pK_d 8.40 ± 0.08), similar to the Ki obtained in homologous inhibition experiments (see below).

Specific [^{3}H]-CGS-21680 binding was inhibited in a concentration-dependent manner by unlabelled CGS-21680 (Fig. 1b), with pK_i value (−log K_i) 8.34 ± 0.10 (K_i 4.53 nM; three experiments). The selective H_3R agonist immepip (30 nM) reduced modestly but significantly $A_{2A}R$ affinity for CGS-21680 (K_i 9.32 nM; pK_i 8.03 ± 0.07; $P = 0.002$, Student's t test, four experiments).

A high density of H_3Rs was previously detected in GP synaptosomal membranes (maximum [^{3}H]-NMHA binding, 1327 ± 79 fmol/mg protein) [16]. The $A_{2A}R$ agonist CGS-21680 (3 and 6 nM) increased in a modest but significant manner: the K_i of the H_3R agonist immepip from 0.63 nM (pK_i 9.20 ± 0.01) to 0.50 and 0.26 nM (pK_i 9.30 ± 0.02; $P < 0.05$ and 9.58 ± 0.02; $P < 0.01$; one-way ANOVA and Tukey's test; three experiments). Figure 1c illustrates the effect of 6 nM CGS-21680.

The stimulatory effect of $A_{2A}R$ activation on depolarization-evoked [3H]-GABA release from GP synaptosomes involves the cAMP/PKA pathway

The Ca^{2+}-dependent [^{3}H]-GABA release from GP synaptosomes induced by depolarization with 20 mM KCl is enhanced by $A_{2A}R$ activation [16]. In this study, the facilitatory effect of CGS-21680 (3 nM, 130.4 ± 3.6% of control values) was mimicked by forskolin (10 μM), a direct adenylyl cyclase activator, and by 8-Bromo-cAMP (500 μM), a membrane permeant cAMP analogue (Fig. 2a, b). In GP slices, electrophysiological studies showed $A_{2A}R$-mediated facilitation of GABA release to depend on the cAMP/PKA pathway [11, 22], and in GP synaptosomes, the PKA inhibitor H-89 (10 μM) prevented the effect of $A_{2A}R$ activation (Fig. 2c). Together, these data indicated that the cAMP/PKA pathway underlies the enhancing effect of $A_{2A}R$ activation on depolarization-evoked [^{3}H]-GABA release from GP synaptosomes.

To test whether the inhibitory action of H_3R activation on the facilitation of [^{3}H]-GABA release induced by the cAMP/PKA pathway was exerted at or downstream cAMP formation, the effect of 8-Bromo-cAMP was evaluated in the presence or absence of the H_3R agonist immepip. Figure 2d shows that H_3R activation had no significant effect on the facilitatory action of 8-Bromo-cAMP on depolarization-evoked [^{3}H]-GABA release from GP synaptosomes.

Effect of $A_{2A}R$ and H_3R activation on depolarization-induced increase in the intracellular Ca^{2+} concentration ([Ca^{2+}]i) of GP synaptosomes

Increasing the concentration of K^+ ions in the perfusing solution from 4.7 to 25 mM resulted in an increase in the [Ca^{2+}]i of GP synaptosomes. Figure 3 shows that in the two-pulse protocol, perfusion with the $A_{2A}R$ agonist CGS-21680 (10 nM) had no effect on the S2/S1 ratio (control 0.985 ± 0.007; CGS-21680 0.979 ± 0.008; $P = 0.656$). The co-perfusion of CGS-21680 and the H_3R agonist immepip (100 nM) also failed to

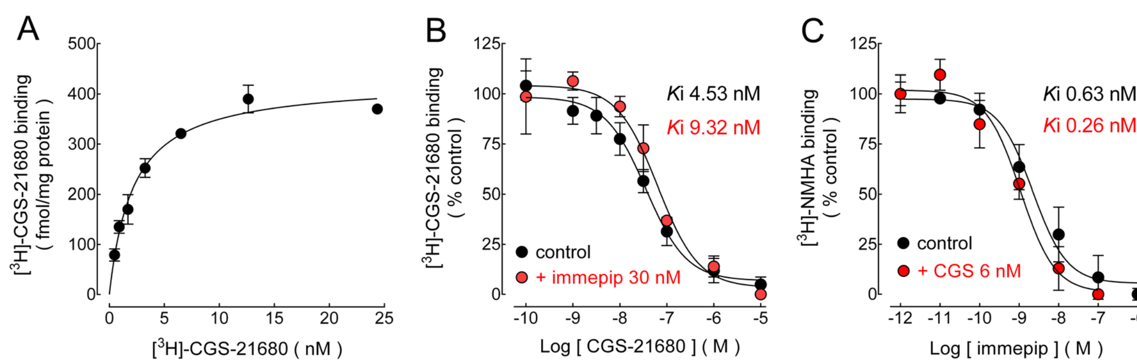

Fig. 1 In GP synaptosomal membranes, H_3R activation decreases $A_{2A}R$ affinity for the agonist CGS-21680, whereas $A_{2A}R$ activation increases H_3R affinity for the agonist immepip. **a** Saturation binding of [^{3}H]-CGS-21680 to A_{2A}Rs. Specific receptor binding was determined by subtracting the binding in the presence of the $A_{2A}R$ antagonist ZM-241385 (10 μM) from total binding. Points are means ± range from duplicates from a single experiment, which was repeated a further four times. The line drawn is the best fit to a hyperbola. Best-fit values for the equilibrium dissociation constant (K_d) and maximum binding (B_{max}) are given in the text. **b** The H_3R agonist immepip (30 nM) decreases the potency of CGS-21680 to inhibit the specific binding of [^{3}H]-CGS-21680 to A_{2A}Rs. **c** The $A_{2A}R$ agonist CGS-21680 (6 nM) increases the potency of immepip to inhibit [^{3}H]-NMHA binding to H_3Rs. For panels **b** and **c**, values are expressed as the percentage of control binding and correspond to means ± SEM from three replicates from representative experiments. The line drawn is the best fit to a logistic equation for a one-site model. The analysis of the K_i and pK_i values calculated from the best-fit IC_{50} estimates is presented in the text

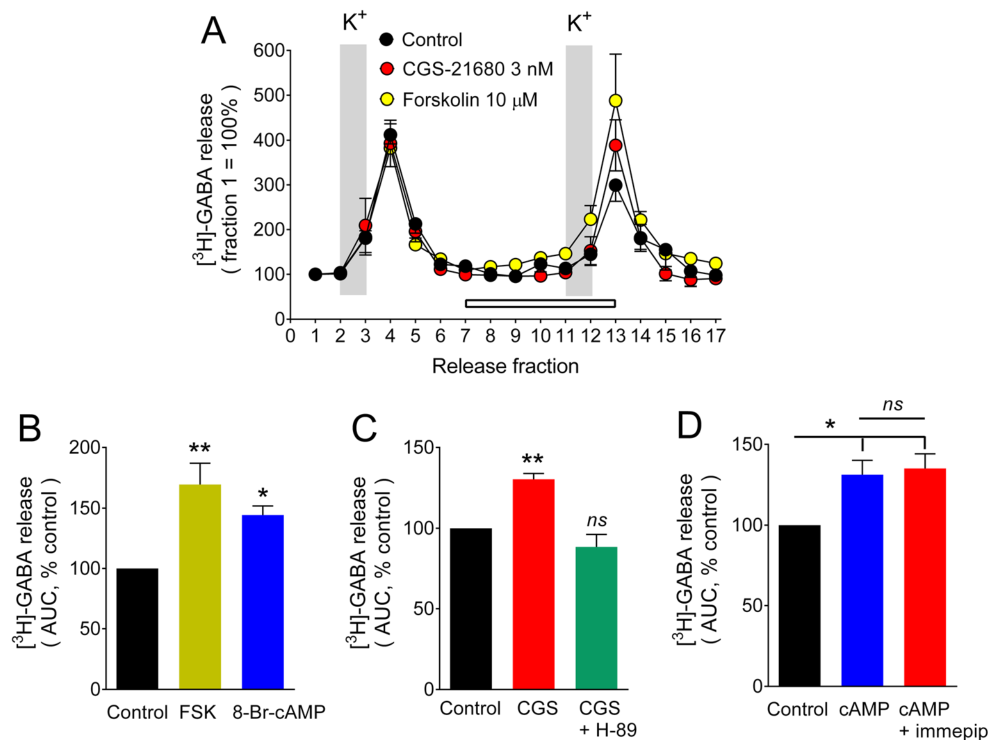

Fig. 2 Involvement of the cAMP/PKA pathway in depolarization-evoked $[^3H]$-GABA release from rat globus pallidus synaptosomes. **a** Illustration of a representative experiment. Labeled synaptosomes were perfused with Krebs-Ringer-Hepes solution and $[^3H]$-GABA release was evoked by raising the K^+ concentration from 4 to 20 mM for the periods indicated by the vertical gray bars. Drugs under test were present for the period indicated by the open bar. Values are expressed as a percentage of $[^3H]$-GABA release in fraction 1 and represent means ± SEM from four to six replicates. **b** Forskolin and 8-Br-cAMP enhance depolarization-evoked $[^3H]$-GABA release. After subtraction of basal release, the area under the release curves for fractions 3–8 (S1) and 12–17 (S2) was determined for each individual chamber and the ratio S2/S1 was calculated. Values are expressed as a percentage of control $[^3H]$-GABA release (no drugs added) and are means ± SEM from three experiments with four to six replicates. The statistical analysis was performed with ANOVA and Dunnett's test. **c** The PKA antagonist H-89 (10 μM) prevented the facilitatory effect of the $A_{2A}R$ agonist CGS-21680 (3 nM) on K^+-evoked $[^3H]$-GABA release. Values are means ± SEM from four experiments. **d** The H_3R agonist immepip (100 nM) did not inhibit the facilitatory effect of 8-Br-cAMP (500 μM) on depolarization-evoked $[^3H]$-GABA release. Values are means ± SEM from five experiments. For panels **b** and **c**, the statistical analysis was performed with ANOVA and Dunnett's test; ns, no significantly different, $*P < 0.05$, $**P < 0.01$ versus control values. For panel **d**, values were compared with ANOVA and Tukey's test; *ns*, no significantly different, $*P < 0.05$

modify the S2/S1 ratio (control 1.023 ± 0.003; CGS-21680/immepip 1.001 ± 0.010; $P = 0.2759$). These results indicated that the effects of $A_{2A}R$ or H_3R activation on GABA release did not involve modulatory actions at voltage-activated Ca^{2+} channels.

Effect of H_3R activation on $A_{2A}R$-induced increases in cAMP accumulation in GP slices

Figure 4a shows that in GP slices, the $A_{2A}R$ agonist CGS-21680 stimulated cAMP accumulation in a concentration-dependent manner (EC_{50} 3.1 nM; maximum effect 281% of basal accumulation at 10 nM). In a different series of experiments, the effect of CGS-21680 (10 nM, 290% of basal accumulation) was reduced by the H_3R agonist immepip (75% inhibition at 100 nM, IC_{50} 8.6 nM; Fig. 4b).

Discussion

In GP slices, $A_{2A}R$ activation facilitates GABA release [9, 12], and electrophysiological studies showed this effect to depend on the cAMP/PKA pathway [11, 22]. We previously reported that H_3Rs are present at high density on GP synaptosomes, where their activation selectively counteracted the facilitatory action of $A_{2A}R$ stimulation on depolarization-evoked $[^3H]$-GABA release [16]. In this work, we analyzed three mechanisms that could underlie the H_3R effect and show that it most likely relies on the inhibition of cAMP formation.

Effect of H_3R activation on the affinity of $A_{2A}Rs$ for the agonist CGS-21680

In transfected cells, H_3Rs form heteromers with dopamine D_1 and D_2 receptors, and in striatal membranes, H_3R activation decreases the affinity of D_2 receptors for agonists [23],

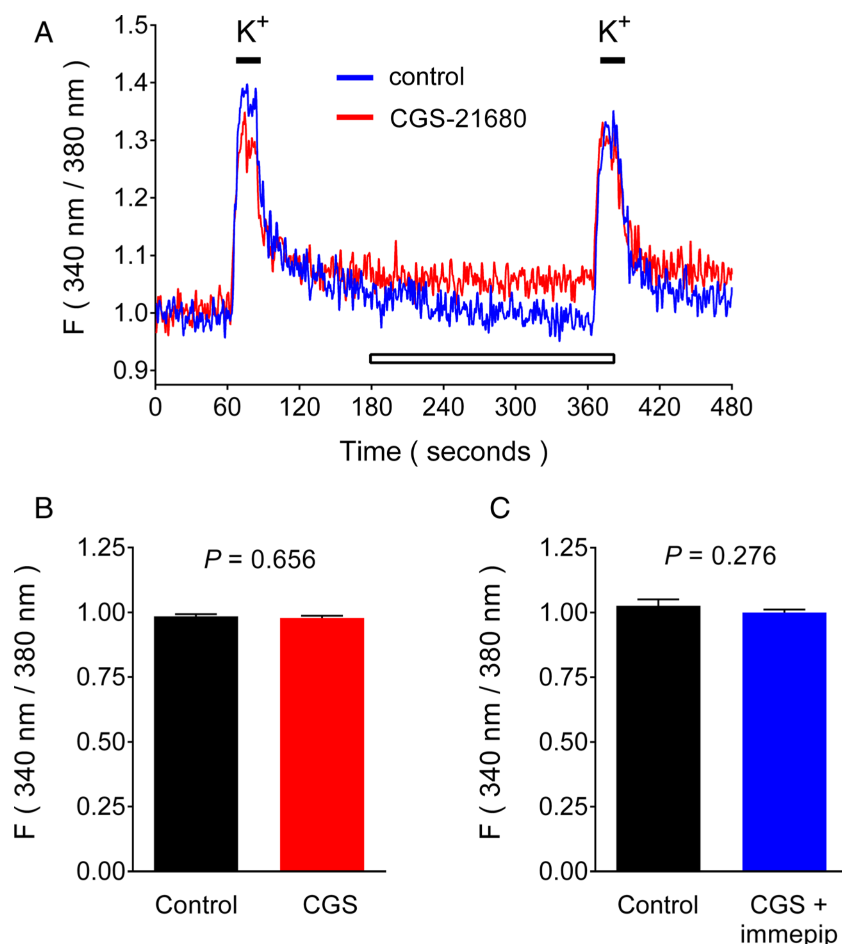

Fig. 3 Lack of effect of $A_{2A}R$ and H_3R activation on the depolarization-induced increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in globus pallidus synaptosomes. **a** Representative traces. Synaptosomes loaded with Fura 2-AM were perfused with Krebs-Ringer-Hepes solution (1 ml/min), and changes in fluorescence were determined by microfluorometry. Drugs under test were present in the solution (open bar) before and during the second depolarization stimulus (25 mM KCl, 20 s, black bars). **b** Lack of effect of the $A_{2A}R$ agonist CGS-21680 (10 nM). The ratios of the second over the first K^+ stimuli (S2/S1) were calculated as described in "Methods" and expressed as means ± SEM from four experiments. AUC, area under the curve. **c** Lack of effect of CGS-21680 (10 nM) and the H_3R agonist immepip (100 nM). Values are means ± SEM from four experiments. The statistical analysis was performed with Student's *t* test

whereas in SK-N-MC cells shifts the coupling of D_1 receptors from $G\alpha_s$ to $G\alpha_{i/o}$ proteins and therefore from stimulation to inhibition of cAMP formation [24]. We recently showed that endogenous and transfected H_3 and A_{2A} receptors form

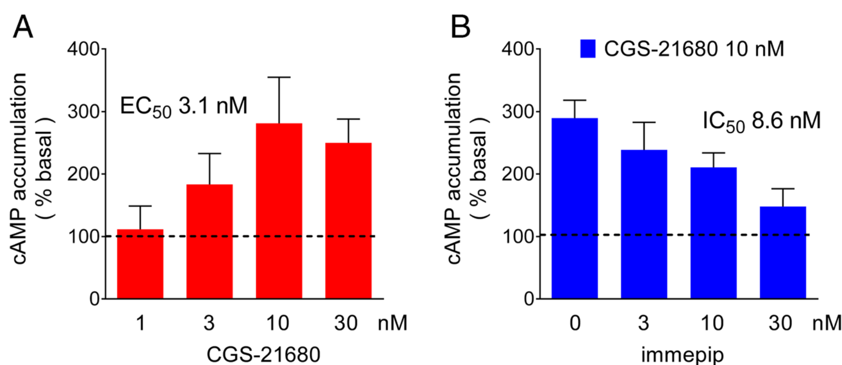

Fig. 4 Effect of $A_{2A}R$ and H_3R activation on cAMP accumulation in rat globus pallidus slices. **a** Stimulation by the $A_{2A}R$ agonist CGS-21680. Slices were incubated with CGS-21680 for 30 min in Krebs-Henseleit solution (1 mM IBMX). Values are means ± SEM from five experiments. **b** Inhibition by H_3R activation of $A_{2A}R$-induced cAMP accumulation. Slices were incubated for 30 min with CGS-21680 (10 nM) in the absence or presence of the indicated concentrations of the H_3R agonist immepip, added 5 min before CGS-21680. Values for the half-stimulatory (EC_{50}, **a**) or half-inhibitory concentrations (IC_{50}, **b**) were obtained by nonlinear regression with GraphPad Prism 5

heterodimers [25], which could therefore underlie the interaction $H_3R/A_{2A}R$ in GABA release.

Our results show that H_3R activation reduces $A_{2A}R$ affinity for the agonist CGS-21680 and, conversely, that $A_{2A}R$ activation increases H_3R affinity for the agonist immepip. Although these data support $H_3R/A_{2A}R$ dimerization in rat GP nerve terminals, the reduction in $A_{2A}R$ affinity (~2-fold) implies a modest effect on receptor occupancy by adenosine, particularly at high concentrations of the modulator.

Fast-scan cyclic voltammetry showed that in anesthetized rats spontaneous adenosine transients yielded 170 and 190 nM for the striatum and the prefrontal cortex [26], whereas in slices, these transients yielded 110, 160, and 240 nM for prefrontal cortex, thalamus, and hippocampus, respectively [27]. $A_{2A}R$ affinity for adenosine approximates 150 nM [1]. Assuming that in GP spontaneous adenosine transients yield the value reported for the striatum (170 nM), $A_{2A}R$ occupancy by adenosine would be 53% and the twofold decrease in affinity for agonists induced by H_3R activation would decrease receptor occupancy to 36%. As mentioned above, this effect would be reduced and eventually abolished at high concentrations of adenosine. This point is illustrated by Fig. 1b, where the shift to the right in the concentration-response curve is observed for concentrations of CGS-21680 between 1 and 30 nM to disappear at concentrations of 100 nM and above. Thus, dimerization alone appears not sufficient to explain the functional interaction between H_3Rs and $A_{2A}Rs$.

Lack of effect of $A_{2A}R$ activation on depolarization-induced increase in $[Ca^{2+}]i$

The coupling of H_3Rs to $G\alpha_{i/o}$ proteins makes likely that their inhibitory effect on neurotransmitter release involves a decrease in depolarization-induced Ca^{2+} entry through N- and P/Q-type voltage-operated channels [28–30]. The $A_{2A}R$-mediated enhancement of both acetylcholine release from striatal synaptosomes and GABA release from hippocampal synaptosomes involves, at least partially, the cAMP/PKA pathway and P-type Ca^{2+} channels [31, 32], and D_1 receptor-mediated facilitation and H_3R-mediated inhibition of GABA release from striatal terminals appear to converge at P-type Ca^{2+} channels [33, 34]. One plausible explanation for the interaction $H_3R/A_{2A}R$ in $[^3H]$-GABA release from striato-pallidal terminals was thus that $G\beta\gamma$ complexes released from $G\alpha_{i/o}$ proteins upon H_3R activation inhibited voltage-activated Ca^{2+} channels whose opening was facilitated by $A_{2A}R$ stimulation, presumably by PKA-mediated phosphorylation. However, in our experiments, the activation of $A_{2A}Rs$ failed to enhance the increase in the $[Ca^{2+}]i$ induced by depolarization in GP synaptosomes, indicating that their facilitatory action on GABA release and therefore the modulatory effect of H_3Rs do not take place at voltage-activated Ca^{2+} channels, at least under the experimental conditions employed in this study. This conclusion is in agreement with the electrophysiological data of Shindou et al. [11], who showed that blocking Ca^{2+} channels with $CdCl_2$ did not prevent the $A_{2A}R$-mediated facilitation of GABA release in GP slices.

$[^3H]$-GABA release and modulation of the cAMP/PKA pathway by $A_{2A}Rs$ and H_3Rs

Shindou et al. [11, 22] showed that the $A_{2A}R$ modulatory effect on GABA release observed in GP slices depended on the activation of the cAMP/PKA pathway, because the adenylyl cyclase activator forskolin increased GABA release and both the adenylyl cyclase inhibitor SQ22536 and the PKA inhibitor H-89 prevented the increase in the frequency of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs). With a neurochemical approach, we show here that forskolin and 8-Bromo-cAMP mimicked the $A_{2A}R$-mediated enhancement of depolarization-induced $[^3H]$-GABA release from GP synaptosomes and that PKA inhibition prevented the $A_{2A}R$ effect (Fig. 2c), supporting the participation of the cAMP/PKA pathway.

The facilitatory effect of A_{2A} receptors on GABA release has been also reported for hippocampal synaptosomes, where CGS-21680 (1–10 nM; apparent EC_{50} 3 nM) enhanced K^+-evoked $[^3H]$-GABA release with maximal facilitation of 34 ± 4% at 10 nM. This effect was mimicked by forskolin (but not by its inactive analogue 1,9-dideoxyforskolin), the cAMP analogues dibutyryl-cAMP and 8-bromo-cAMP, and the phosphodiesterase inhibitor rolipram. Furthermore, the effect of a submaximal concentration of CGS-21680 (1 nM) was significantly occluded by dibutyryl-cAMP, 8-bromo-cAMP, and forskolin and was potentiated by the phosphodiesterase inhibitor rolipram [32]).

In regard to other neurotransmitters, for the neuromuscular transmission, the facilitation by methylprednisolone of exocytosis and $[^3H]$-acetylcholine release involves the activation of presynaptic A_{2A} receptors by endogenous adenosine leading to synaptic vesicle redistribution, and the methylprednisolone effect was markedly reduced by PKA inhibition [35]. In differentiated human neuroblastoma SH-SY5Y cells, the activation of endogenous A_{2A} receptors facilitates depolarization-evoked $[^3H]$-noradrenaline release, and this effect was also prevented by PKA inhibition [36]. This information supports that the facilitatory effect of A_{2A} receptors on neurotransmitter release is mainly mediated by the cAMP/PKA pathway.

In this work, H_3R activation failed to inhibit the effect of 8-Bromo-cAMP on GABA release (Fig. 2d), indicating that the H_3R action on $A_{2A}R$-mediated enhancement of GABA release is exerted at the level of cAMP formation. This hypothesis is supported by the H_3R-mediated inhibition of $A_{2A}R$-induced cAMP accumulation observed in GP slices (Fig. 4). Because $A_{2A}R$ activation had no effect on the $[Ca^{2+}]i$ in GP synaptosomes (Fig. 3), the cAMP/PKA pathway may thus act

downstream Ca^{2+} entry, for example on exocytosis proteins such as synapsin 1, SNAP-25, rabfilin 3A, syntaxin, and RIM1a/2a, whose phosphorylation by PKA leads to redistribution of synaptic vesicles [35, 37, 38]. In this regard, in neurons of rat medulla oblongata in primary culture, $A_{2A}R$ activation enhanced exocytosis and the phosphorylation of synapsin I and the latter effect was prevented by the PKA inhibition [38].

Conclusion

The GP has emerged as a key point in the control of the basal ganglia motor output [5, 6]. In this work, we showed that the opposite effects of $A_{2A}Rs$ and H_3Rs on GABA release from striato-pallidal afferents rely on the stimulation and inhibition of the cAMP/PKA pathway, respectively. Recent work shows that cAMP formation in striato-pallidal neurons increases their spiking activity leading to inhibition of the spontaneous firing of GP neurons and reduced motor activity [39]. Through the activation of presynaptic H_3Rs, histamine could therefore contribute to the regulation of the activity of GP neurons and thus of basal ganglia function.

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Author contributions G.-E.M.-F., E.J.G., and J.-A.A.-M. designed the study. G.-E.M.-F., J.E.-S., R.G.-P., U.G.-H., and N.R.-R. conducted experiments. G.-E.M.-F. and J.-A.A.-M. performed data analysis. G.-E.M.-F. and J.-A.A.-M. wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare they do not have any actual or potential conflict of interest.

Ethical approval All procedures were approved by the Cinvestav Animal Care Committee and followed the guidelines for the care and use of laboratory animals issued by the National Institutes of Health (NIH Publications No. 8023, revised 1978) and the Mexican Council for Animal Care (NOM-062-ZOO-1999).

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